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(54) Tide: MAMMALIAN MELANOCYTE STIMULATING HORMONE RECEPTORS AND USES

### (57) Abstract

The present invention relates to a mammalian melanocyte stimulating hormone receptor. The invention is directed toward the isolation, characterization and pharmacological use of mammalian melanocyte stimulating hormone receptor, the gene corresponding to this receptor, a recombinant eukaryotic expression construct capable of expressing a mammalian melanocyte stimulating hormone receptor in cultures of transformed eukaryotic cells and such cultures of transformed eukaryotic cells that synthesize mammalian melanocyte stimulating hormone receptor. The invention also provides methods for screening MSHR agonists and antagonists in vitro using preparations of receptor from such cultures of eukaryotic cells transformed with a recombinant eukaryotic expression construct comprising the MSHR receptor gene. The invention specifically provides human and mouse MSHR genes.

300 NDP-MSH 250 aMSH **ACTH** cAMP (%Basal) 200 150 100 50 10 -13 10 -12 10 -11 10 -8 10-7 10 Log [Hormone] (M)

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# MAMMALIAN MELANOCYTE STIMULATING HORMONE RECEPTORS AND USES

# BACKGROUND OF THE INVENTION

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This invention was made with government support under 1R01DK41921-03, 1R01DK43859-01, and 1P01DK44239-10A1 by the National Institutes of Health. The government has certain rights in the invention.

# 10 1. Field of the Invention

This invention relates to melanocyte stimulating hormone receptors from mammalian species and the genes corresponding to such receptors. Specifically, the invention relates to the isolation, cloning and sequencing of a human melanocyte stimulating hormone receptor gene. The invention also relates to the isolation, cloning and sequencing of a mouse melanocyte stimulating hormone The invention relates to the construction of eukaryotic receptor gene. recombinant expression constructs capable of expressing these melanocyte stimulating hormone receptors in cultures of transformed eukaryotic cells, and the production of the melanocyte stimulating hormone receptor in such cultures. The invention relates to the use of such cultures of transformed eukaryotic cells to produce homogeneous compositions of such melanocyte stimulating hormone The invention also provides cultures of such cells producing receptors. melanocyte stimulating hormone receptor for the characterization of novel and useful drugs. Antibodies against and epitopes of these melanocyte stimulating hormone receptor proteins are also provided by the invention.

# 2. Background of the Invention

The proopiomelanocortin (POMC) gene product is processed to produce a large number of biologically active peptides. Two of these peptides,  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ MSH), and adrenocorticotropic hormone (ACTH) have well understood roles in control of melanocyte and adrenocortical function, respectively. Both of these hormones, however, are found in a variety of forms with unknown functions. The melanocortin peptides also have a diverse

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array of biological activities in other tissues, including the brain, and immune system, and bind to specific receptors there with a distinct pharmacology [see, Hanneman et al., in Peptide Hormone as Prohormones, G. Martinez, ed. (Ellis Horwood Ltd.: Chichester, UK) pp. 53-82; DeWied & Jolles, 1982, Physiol. Rev. 62: 976-1059 for reviews].

A complete understanding of these peptides and their diverse biological activities requires the isolation and characterization of their corresponding receptors. Some biochemical studies have been reported on the prior art.

Shimuze, 1985, Yale J. Biol. Med. <u>58</u>: 561-570 discusses the physiology of melanocyte stimulating hormone.

Tatro & Reichlin, 1987, Endocrinology <u>121</u>: 1900-1907 disclose that MSH receptors are widely distributed in rodent tissues.

Solca et al., 1989, J. Biol. Chem. <u>264</u>: 14277-14280 disclose the molecular weight characterization of mouse and human MSH receptors linked to radioactively and photoaffinity labeled MSH analogues.

Siegrist et al., 1991, J. Receptor Res. 11: 323-331 disclose the quantification of receptors on mouse melanoma tissue by receptor autoradiography.

The present invention comprises a human melanocyte stimulating hormone receptor gene, the nucleotide sequence of this gene and the deduced amino acid sequence of its cognate protein, a homogeneous composition of the melanocyte stimulating hormone receptor, nucleic acid hybridization probes and a method for determining the tissue distribution of expression of the gene, a recombinant expression construct capable of expressing the gene in cultures of transformed eukaryotic cells, and such cultures of transformed eukaryotic cells useful in the characterization of novel and useful drugs. The present invention also comprises the homologue of the human melanocyte stimulating hormone receptor gene from the mouse.

### **DESCRIPTION OF THE DRAWINGS**

Figure 1 illustrates the nucleotide sequence of the mouse (SEQ ID NO:3) and human (SEQ ID NO:5) melanocyte stimulating hormone receptor.

Figure 2 presents an amino acid sequence comparison between the mouse and human melanocyte stimulating hormone receptor proteins.

Figure 3 illustrates binding of melanocyte stimulating hormone receptor agonists to mouse melanocyte stimulating hormone receptor expressed in human 293 cells.

Figure 4 illustrates the tissue distribution of human (Panel A) and mouse

(Panel B) melanocyte stimulating hormone receptor gene expression by Northern blot hybridization.

### SUMMARY OF THE INVENTION

The present invention relates to the cloning, expression and functional characterization of mammalian melanocyte stimulating hormone receptor (MSH<sup>R</sup>) genes. The invention comprises the nucleotide sequence of these genes encoding the mammalian MSH<sup>R</sup>s and the deduced amino acid sequences of the cognate proteins, as well as tissue distribution patterns of expression of these genes.

In particular, the present invention is directed toward the isolation, characterization and pharmacological use of the human MSH<sup>R</sup>, the gene corresponding to this receptor, a nucleic acid hybridization probe comprising DNA sequences of the human MSH<sup>R</sup>, a recombinant eukaryotic expression construct capable of expressing the human MSH<sup>R</sup> in cultures of transformed eukaryotic cells and such cultures of transformed eukaryotic cells that synthesize the human MSH<sup>R</sup>, a homogeneous composition of the human MSH<sup>R</sup>, and antibodies against and epitopes of the human MSH<sup>R</sup>.

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The present invention is also directed toward the isolation, characterization and pharmacological use of the mouse MSH<sup>R</sup>, the gene corresponding to this receptor, a nucleic acid hybridization probe comprising DNA sequences of the mouse MSH<sup>R</sup>, a recombinant eukaryotic expression construct capable of expressing the mouse MSH<sup>R</sup> in cultures of transformed eukaryotic cells and such cultures of transformed eukaryotic cells that synthesize the mouse MSH<sup>R</sup>, a homogeneous composition of the mouse MSH<sup>R</sup>, and antibodies against and epitopes of the mouse MSH<sup>R</sup>.

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It is an object of the invention to provide a nucleic acid comprising a nucleotide sequence encoding a mammalian MSH<sup>R</sup>. In a preferred embodiment of the invention, the nucleotide sequence encodes the human MSH<sup>R</sup>. In another preferred embodiment, the nucleotide sequence encodes the mouse MSH<sup>R</sup>.

The present invention includes a nucleic acid comprising a nucleotide sequence encoding a human MSH<sup>R</sup> receptor derived from a DNA molecule isolated from a human genomic library (SEQ ID NO:5). In this embodiment of the invention, the nucleotide sequence includes 1635 nucleotides of the human MSH<sup>R</sup> gene comprising 953 nucleotides of coding sequence, 462 nucleotides of

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5' untranslated sequence and 220 nucleotides of 3' untranslated sequence.

The present invention also includes a nucleic acid comprising a nucleotide sequence encoding a mouse MSH<sup>R</sup> derived from a cDNA molecule isolated from a CDNA library constructed with RNA from mouse Cloudman melanoma cells (SEQ ID NO:3). In this embodiment of the invention, the nucleotide sequence includes 1260 nucleotides of the mouse MSH<sup>R</sup> gene comprising 947 nucleotides of coding sequence, 15 nucleotides of 5' untranslated sequence and 298 nucleotides of 3' untranslated sequence.

The invention includes nucleic acids comprising the nucleotide sequences of mammalian MSH<sup>R</sup>s, most preferably mouse and human MSH<sup>R</sup>s (SEQ ID NOs:3&5), and includes allelic variations of these nucleotide sequences and the corresponding MSH<sup>R</sup> molecule, either naturally occurring or the product of *in vitro* chemical or genetic modification, each such variant having essentially the same nucleotide sequence as the nucleotide sequence of the corresponding MSH<sup>R</sup> disclosed herein, wherein the resulting MSH<sup>R</sup> molecule has substantially the same biological properties as the MSH<sup>R</sup> molecule corresponding to the nucleotide sequence described herein. The term "substantially homologous to" as used in this invention encompasses such allelic variability as described in this paragraph.

The invention also includes a protein comprised of a predicted amino acid sequence for the mouse (SEQ ID NO:4) and human (SEQ ID NO:6) MSH<sup>R</sup> deduced from the nucleotide sequence comprising the complete coding sequence of the mouse (SEQ ID NO:3) and human (SEQ ID NO:5) MSH<sup>R</sup> gene as described herein.

In another aspect, the invention comprises a homogeneous composition of a 35.3 kilodalton mouse MSH<sup>R</sup> or derivative thereof, wherein the amino acid sequence of the MSH<sup>R</sup> or derivative thereof comprises the mouse MSH-R sequence shown in Figure 2 (SEQ ID NO:4).

In another aspect, the invention comprises a homogeneous composition of a 34.7 kilodalton human MSH<sup>R</sup> or derivative thereof, wherein the amino acid sequence of the MSH<sup>R</sup> or derivative thereof comprises the human MSH-R sequence shown in Figure 2 (SEQ ID NO:6).

This invention provides both nucleotide and amino acid probes derived from these sequences. The invention includes probes isolated from either cDNA or genomic DNA clones, as well as probes made synthetically with the sequence information derived therefrom. The invention specifically includes but is not limited to oligonucleotide, nick-translated, random primed, or *in vitro* amplified probes made using cDNA or genomic clone embodying the invention, and oligonucleotide and other synthetic probes synthesized chemically using the nucleotide sequence information of cDNA or genomic clone embodiments of the invention.

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It is a further object of this invention to provide sequences of mammalian MSH<sup>R</sup>, preferably the mouse or human MSH<sup>R</sup>, for use as nucleic acid hybridization probes to determine the pattern, amount and extent of expression of this receptor in various tissues of mammals, including humans. It is also an object of the present invention to provide nucleic acid hybridization probes derived from the sequences of the mouse or human MSH<sup>R</sup> to be used for the detection and diagnosis of genetic diseases. It is an object of this invention to provide nucleic acid hybridization probes derived from the DNA sequences of the mouse or human MSH<sup>R</sup> to be used for the detection of novel related receptor genes.

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The present invention also includes synthetic peptides made using the nucleotide sequence information comprising cDNA or genomic clone embodiments of the invention. The invention includes either naturally occurring or synthetic peptides which may be used as antigens for the production of MSH<sup>R</sup>-specific antibodies, or used for competitors of the MSH<sup>R</sup> molecule for drug binding, or to be used for the production of inhibitors of the binding of agonists or antagonists or analogues thereof to MSH<sup>R</sup> molecule.

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The present invention also provides antibodies against and epitopes of mammalian MSH<sup>R</sup>s, preferably mouse or human MSH<sup>R</sup> proteins. It is an object of the present invention to provide antibodies that is immunologically reactive to a mammalian MSH<sup>R</sup> protein. It is a particular object of the invention to provide a monoclonal antibodies to mammalian MSH<sup>R</sup> protein, most preferably mouse or

human MSH<sup>R</sup> protein.

It is also an object of the present invention to provide a hybridoma cell line that produces such an antibody. It is a particular object of the invention to provide a hybridoma cell line that is the result of fusion between a non-immunoglobulin producing mouse myeloma cell line and spleen cells derived from a mouse immunized with a human cell line which expresses MSH<sup>R</sup> antigen. The present invention also provides a hybridoma cell line that produces such an antibody, and that can be injected into a living mouse to provide an ascites fluid from the mouse that is comprised of such an antibody.

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The present invention also provides a pharmaceutical composition comprising a therapeutically effective amount of a monoclonal antibody that is immunologically reactive to a mammalian MSH<sup>R</sup>, preferably a mouse or human MSH<sup>R</sup>, and in a pharmaceutically acceptable carrier.

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It is a further object of the present invention to provide an epitope of a mammalian MSH<sup>R</sup> protein wherein the epitope is immunologically reactive to an antibody specific for the mammalian MSH<sup>R</sup>. In preferred embodiments, the epitope is derived from mouse of human MSH<sup>R</sup> protein.

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It is another object of the invention to provide a chimeric antibody that is immunologically reactive to a mammalian MSH<sup>R</sup> protein. In a preferred embodiment, the chimeric antibody is a monoclonal antibody. In a preferred embodiment, the MSH<sup>R</sup> is a mouse or human MSH<sup>R</sup>.

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The present invention provides a recombinant expression construct comprising the nucleotide sequence of a mammalian MSH<sup>R</sup>, preferably the mouse or human MSH<sup>R</sup> and sequences sufficient to direct the synthesis of mouse or human MSH<sup>R</sup> in cultures of transformed eukaryotic cells. In a preferred embodiment, the recombinant expression construct is comprised of plasmid sequences derived from the plasmid pcDNAI/neo and cDNA or genomic DNA of mouse or human MSH<sup>R</sup> gene. This invention includes a recombinant expression construct comprising essentially the nucleotide sequences of genomic or cDNA clones of mouse or human MSH<sup>R</sup> in an embodiment that provides for their expression in cultures of transformed eukaryotic cells.

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It is also an object of this invention to provide cultures of transformed eukaryotic cells that have been transformed with such a recombinant expression construct and that synthesize mammalian, preferably mouse or human, MSH<sup>R</sup> protein. In a preferred embodiment, the invention provides human 293 cells that synthesize mouse MSH<sup>R</sup>. In an additional preferred embodiment, the invention provides human 293 cells that synthesize human MSH<sup>R</sup> protein.

The present invention also includes protein preparations of mammalian, preferably mouse or human MSH<sup>R</sup>, and preparations of membranes containing mammalian MSHR, derived from cultures of transformed eukaryotic cells. In a preferred embodiment, cell membranes containing mouse MSH<sup>R</sup> protein are isolated from 293 cell cultures transformed with a recombinant expression construct that directs the synthesis of mouse MSH<sup>R</sup>. In another preferred embodiment, cell membranes containing human MSH<sup>R</sup> protein are isolated from 293 cell cultures transformed with a recombinant expression construct that directs It also an object of this invention to provide the synthesis of human MSH<sup>R</sup>. mammalian, preferably mouse or human MSHR for use in the in vitro screening of novel adenosine agonist and antagonist compounds. In a preferred embodiment, membrane preparations containing the mouse MSH<sup>R</sup>, derived from cultures of transformed eukaryotic cells, are used to determine the drug dissociation properties of various novel adenosine agonist and antagonist compounds in vitro. In another preferred embodiment, membrane preparations containing the human MSH<sup>R</sup>, derived from cultures of transformed eukaryotic cells, are used to determine the drug dissociation properties of various novel adenosine agonist and antagonist compounds in vitro. These properties are then used to characterize such novel compounds by comparison to the binding properties of known mouse or human MSHR agonists and antagonists.

The present invention will also be useful for the *in vivo* detection of analogues of agonists or antagonists of MSH<sup>R</sup>, known or unknown, either naturally occurring or as the embodiments of a drug.

It is an object of the present invention to provide a method for the quantitative detection of agonists or antagonists, or analogues thereof, of MSH<sup>R</sup>,

known or unknown, either naturally occurring or as the embodiments of a drug. It is an additional object of the invention to provide a method to detect such agonists, antagonists, or analogues thereof in blood, saliva, semen, cerebrospinal fluid, plasma, lymph, or any other bodily fluid.

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Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "melanocyte stimulating hormone receptor" as used herein refers to proteins substantially homologous to, and having substantially the same biological activity as, the protein coded for by the nucleotide sequence depicted in Figure 1 (SEQ ID NO:3). This definition is intended to encompass natural allelic variations in the melanocyte stimulating hormone receptor sequence. Cloned genes of the present invention may code for MSH<sup>R</sup>s of any species of origin, including, for example, mouse, rat, rabbit, cat, and human, but preferably code for receptors of mammalian, most preferably mouse and human, origin.

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Nucleic acid hybridization probes provided by the invention comprise DNA sequences that are substantially homologous to the DNA sequences in Figure 1A (SEQ ID NO:3) and 1B (SEQ ID NO:5). Nucleic acid probes are useful for detecting MSH<sup>R</sup> gene expression in cells and tissues using techniques well-known in the art, including but not limited to Northern blot hybridization, in situ hybridization and Southern hybridization to reverse transcriptase - polymerase chain reaction product DNAs. The probes provided by the present invention, including oligonucleotides probes derived therefrom, are useful are also useful for Southern hybridization of mammalian, preferably human, genomic DNA for screening for restriction fragment length polymorphism (RFLP) associated with certain genetic disorders.

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The production of proteins such as the MSH<sup>R</sup> from cloned genes by genetic engineering is well known. See, e.g., U.S. Patent No. 4,761,371 to Bell et al. at Col. 6 line 3 to Col. 9 line 65. (The disclosure of all U.S. patent references cited herein is to be incorporated herein by reference.) The discussion which follows is accordingly intended as an overview of this field, and is not intended to reflect the full state of the art.

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DNA which encodes the MSH<sup>R</sup> may be obtained, in view of the instant disclosure, by chemical synthesis, by screening reverse transcripts of mRNA from appropriate cells or cell line cultures, by screening genomic libraries from appropriate cells, or by combinations of these procedures, as illustrated below. Screening of mRNA or genomic DNA may be carried out with oligonucleotide

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probes generated from the MSH<sup>R</sup> gene sequence information provided herein. Probes may be labeled with a detectable group such as a fluorescent group, a radioactive atom or a chemiluminescent group in accordance with know procedures and used in conventional hybridization assays, as described in greater detail in the Examples below. In the alternative, MSH<sup>R</sup> gene sequences may be obtained by use of the polymerase chain reaction (PCR) procedure, with the PCR oligonucleotide primers being produced from the MSH<sup>R</sup> gene sequence provided herein. See U.S. Patent Nos. 4,683,195 to Mullis et al. and 4,683,202 to Mullis.

The MSH<sup>R</sup> may be synthesized in host cells transformed with a recombinant expression construct comprising a DNA sequence encoding the MSH<sup>R</sup>. Such a recombinant expression construct can also be comprised of a vector that is a replicable DNA construct. Vectors are used herein either to amplify DNA encoding the MSHR and/or to express DNA which encodes the MSH<sup>R</sup>. For the purposes of this invention, a recombinant expression construct is a replicable DNA construct in which a DNA sequence encoding the MSHR is operably linked to suitable control sequences capable of effecting the expression of the MSHR in a suitable host. The need for such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants.

Vectors useful for practicing the present invention include plasmids, viruses (including phage), retroviruses, and integratable DNA fragments (i.e., fragments integratable into the host genome by homologous recombination). The vector replicates and functions independently of the host genome, or may, in some instances, integrate into the genome itself. Suitable vectors will contain replicon and control sequences which are derived from species compatible with the

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intended expression host. A preferred vector is the plasmid pcDNAI/neo. Transformed host cells are cells which have been transformed or transfected with recombinant expression constructs made using recombinant DNA techniques and comprising a mammalian MSH<sup>R</sup>. Transformed host cells may ordinarily express the mammalian MSH<sup>R</sup>, but host cells transformed for purposes of cloning or amplifying nucleic acid hybridization probe DNA need not express the receptor. When expressed, the mammalian MSH<sup>R</sup> will typically be located in the host cell membrane.

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DNA regions are operably linked when they are functionally related to each other. For example: a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of leaders sequences, contiguous and in the same translational reading frame.

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Cultures of cells derived from multicellular organisms are a desirable host for recombinant MSH<sup>R</sup> synthesis. In principal, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. However, mammalian cells are preferred, as illustrated in the Examples. Propagation of such cells in cell culture has become a routine procedure. See Tissue Culture, Academic Press, Kruse & Patterson, editors (1973). Examples of useful host cell lines are human 293 cells, VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and WI138, BHK, COS-7, CV, and MDCK cell lines. Human 293 cells are preferred. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located upstream from the gene to be expressed, along with a ribosome binding site, RNA splice sites (if introncontaining genomic DNA is used), a polyadenylation site, and a transcriptional termination sequence.

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An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral source (e.g., polyoma, adenovirus, VSV, or MPV), or may be provided by the host cell chromosomal replication mechanism. If the vector is

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integrated into the host cell chromosome, the latter may be sufficient.

The invention provides homogeneous compositions of mammalian MSH<sup>R</sup> protein produced by transformed eukaryotic cells as provided herein. Such homogeneous compositions are intended to be comprised of mammalian MSH<sup>R</sup> protein that comprises 90% of the protein in such homogeneous composition.

Mammalian MSH<sup>R</sup> protein made from cloned genes in accordance with the present invention may be used for screening agonist compounds for MSH<sup>R</sup> activity, or for determining the amount of a MSH<sup>R</sup> agonist or antagonist drug in a solution (e.g., blood plasma or serum). For example, host cells may be transformed with a recombinant expression construct of the present invention, MSH<sup>R</sup> expressed in that host, the cells lysed, and the membranes from those cells used to screen compounds for MSH<sup>R</sup> binding activity. Competitive binding assays in which such procedures may be carried out are well known in the art. By selection of host cells which do not ordinarily express MSH<sup>R</sup>s, pure preparations of membranes containing MSH<sup>R</sup>s can be obtained. Further, MSH<sup>R</sup> agonists and antagonists can be identified by transforming host cells with vectors of the present invention. Membranes obtained from such cells can be used in binding studies wherein the drug dissociation activity is monitored.

The recombinant expression constructs of the present invention are useful in molecular biology to transform cells which do not ordinarily express the MSH<sup>R</sup> to thereafter express this receptor. Such cells are useful as intermediates for making cell membrane preparations useful for receptor binding assays, which are in turn useful for drug screening. Further, genes and vectors comprising the recombinant expression construct of the present invention are useful in gene therapy. For such purposes, retroviral vectors as described in U.S. Patent No. 4,650,764 to Temin & Watanabe or U.S. Patent No. 4,861,719 to Miller may be employed. Cloned genes of the present invention, or fragments thereof, may also be used in gene therapy carried out homologous recombination or site-directed mutagenesis. See generally Thomas & Capecchi, 1987, Cell 51: 503-512; Bertling, 1987, Bioscience Reports 7: 107-112; Smithies et al., 1985, Nature 317: 230-234.

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Oligonucleotides of the present invention are useful as diagnostic tools for probing MSH receptor gene expression in tissues. For example, tissues can be probed in situ with oligonucleotide probes carrying detectable groups by conventional autoradiography techniques, as explained in greater detail in the Examples below, to investigate native expression of this receptor or pathological conditions relating thereto. Further, chromosomes can be probed to investigate the presence or absence of the MSH<sup>R</sup> gene, and potential pathological conditions related thereto, as also illustrated by the Examples below.

The invention also provides antibodies that are immunologically reactive to a mammalian MSH<sup>R</sup>. The antibodies provided by the invention can be raised in animals by inoculation with cells that express a mammalian MSH<sup>R</sup> or epitopes of a mammalian MSH<sup>R</sup> using methods well known in the art. Animals that can be used for such inoculations include individuals from species comprising cows, sheep, pigs, mice, rats, rabbits, hamsters, goats and primates. Preferred animals for inoculation are rodents (including mice, rats, hamsters) and rabbits. The most preferred animal is the mouse.

Cells that can be used for such inoculations, or for any of the other means used in the invention, include any cell line which naturally expresses a mammalian MSH<sup>R</sup>, or any cell or cell line that expresses a mammalian MSH<sup>R</sup> or any epitope therein as a result of molecular or genetic engineering, or that has been treated to increase the expression of a mammalian MSH<sup>R</sup> by physical, biochemical or genetic means. Preferred cells are human cells, most preferably human 293 cells that have been transformed with a recombinant expression construct comprising DNA sequences encoding a mammalian MSH<sup>R</sup> and that express the mammalian MSH<sup>R</sup> gene product.

The present invention provides monoclonal antibodies that are immunologically reactive with an epitope that is a mammalian MSH<sup>R</sup> present on the surface of mammalian cells, preferably human or mouse cells. These antibodies are made using methods and techniques well known to those of skill in the art.

Monoclonal antibodies provided by the present invention are produced by

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hybridoma cell lines, that are also provided by the invention and that are made by methods well known in the art. Hybridoma cell lines are made by fusing individual cells of a myeloma cell line with spleen cells derived from animals immunized with cells expressing a mammalian MSHR, including human cells, as described above. The myeloma cell lines used in the invention include lines derived from myelomas of mice, rats, hamsters, primates and humans. Preferred myeloma cell lines are from mouse, and the most preferred mouse myeloma cell line is P3X63-Ag8.653. The animals from whom spleens are obtained after immunization are rats, mice and hamsters, preferably mice, most preferably Balb/c mice. Spleen cells and myeloma cells are fused using a number of methods well known in the art, including but not limited to incubation with inactivated Sendai virus and incubation in the presence of polyethylene glycol (PEG). The most preferred method for cell fusion is incubation in the presence of a solution of 45% (w/v) PEG-1450. Monoclonal antibodies produced by hybridoma cell lines can be harvested from cell culture supernatant fluids from in vitro cell growth; alternatively, hybridoma cells can be injected subcutaneously and/or into the peritoneal cavity of an animal, most preferably a mouse, and the monoclonal antibodies obtained from blood and/or ascites fluid.

Monoclonal antibodies provided by the present invention can also be produced by recombinant genetic methods well known to those of skill in the art, and the present invention encompasses antibodies made by such methods that are immunologically reactive with an epitope of a mammalian MSH<sup>R</sup>.

The present invention encompasses fragments of the antibody that are immunologically reactive with an epitope of a mammalian MSH<sup>R</sup>. Such fragments can be produced by any number of methods, including but not limited to proteolytic cleavage, chemical synthesis or preparation of such fragments by means of genetic engineering technology. The present invention also encompasses single-chain antibodies that are immunologically reactive with an epitope of a mammalian MSH<sup>R</sup> made by methods known to those of skill in the art.

The present invention also encompasses an epitope of a mammalian MSH<sup>R</sup> that is comprised of sequences and/or a conformation of sequences present in the

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mammalian MSH<sup>R</sup> molecule. This epitope may be naturally occurring, or may be the result of proteolytic cleavage of the mammalian MSH<sup>R</sup> molecule and isolation of an epitope-containing peptide or may be obtained by synthesis of an epitope-containing peptide using methods well known to those skilled in the art. The present invention also encompasses epitope peptides produced as a result of genetic engineering technology and synthesized by genetically engineered prokaryotic or eukaryotic cells.

The invention also includes chimeric antibodies, comprised of immunologically reactive light chain and heavy chain peptides to an epitope that is a mammalian MSH<sup>R</sup>. The chimeric antibodies embodied in the present invention include those that are derived from naturally occurring antibodies as well as chimeric antibodies made by means of genetic engineering technology well known to those of skill in the art.

The Examples which follow are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

### **EXAMPLE 1**

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# Isolation of an aMSH Receptor Probe by Random PCR Amplification of Human Melanoma cDNA Using <u>Degenerate Oligonucleotide Primers</u>

In order to clone novel G-protein coupled receptors, human melanoma cDNA was used as template for a polymerase chain reaction (PCR)-based random cloning experiment. PCR was performed using a pair of degenerate oligonucleotide primers corresponding to the putative third and sixth transmembrane regions of G-protein coupled receptors (Libert et al., 1989, Science 244: 569-72; Zhou et al., 1990, Nature 347: 76-80). The PCR products obtained in this experiment were characterized by nucleotide sequencing. Two novel sequences representing novel G-protein-coupled receptors were identified.

PCR amplification was performed as follows. Total RNA was isolated from a human melanoma tumor sample by the guanidinium thiocyanate method

(Chirgwin et al., 1979, Biochemistry 18: 5294-5299). Double-stranded cDNA was synthesized from total RNA with murine reverse transcriptase (BRL, Gaithersburg, MD) by oligo-dT priming [Maniatis et al., Molecular Cloning: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY), 1990]. The melanoma cDNA mixture was then subjected to 45 cycles of PCR amplification using 500 picomoles of degenerate oligonucleotide primers having the following sequence:

Primer III (sense):

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GAGTCGACCTGTG(C/T)G(C/T)(C/G)AT(C/T)(A/G)CIIT(G/T)GAC(C/A)G(C/G)T AC

(SEQ ID NO:1)

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Primer VI (antisense):

CAGAATTCAG(T/A)AGGGCAICCAGCAGAI(G/C)(G/A)(T/C)GAA (SEQ ID NO:2)

in 100  $\mu$ l of a solution containing 50 mM Tris-HCl (pH 8.3), 2.5 mM MgCl<sub>2</sub>, 0.01% gelatin, 200  $\mu$ M each dNTP, and 2.5 units of Taq polymerase (Saiki et al., 1988, Science 239: 487-491). These primers were commercially synthesized by Research Genetics Inc. (Huntsville, AL). Each PCR amplification cycle consisted of incubations at 94°C for 1 min (denaturation), 45°C for 2 min (annealing), and 72°C for 2 min (extension).

Amplified products of the PCR reaction were extracted with phenol/chloroform and precipitated with ethanol. After digestion with *EcoRI* and *SalI*, the PCR products were separated on a 1.2% agarose gel. A slice of this gel, corresponding to PCR products of 300 basepairs (bp) in size, was cut out and purified using glass beads and sodium iodide, and the insert was then cloned into a pBKS cloning vector (Stratagene, LaJolla, CA).

A total of 172 of such pBKS clones containing inserts were sequenced using Sequenase (U. S. Biochemical Corp., Cleveland, OH) by the dideoxynucleotide chain termination method (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA 74: 5463-5467). Two types of sequences homologous to other

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G-protein coupled receptors were identified.

### **EXAMPLE 2**

### Isolation and Sequence Analysis of Mouse aMSH Receptor cDNA

Probes isolated in Example 1 was used to screen a Cloudman melanoma cDNA library in order to isolate a full-length cDNA corresponding to the cloned probe. One clone was isolated from a library of 5 x 10<sup>6</sup> clones screened as described below. This clone contained an insert of 2.6 kilobases (kb). The nucleotide sequence of the complete coding region was determined, as shown in Figure 1A (SEQ ID NO:3).

The PCR probe was labeled by the random-priming method (Stratagene PrimeIt, #300387, LaJolla, CA) and used to screen a Cloudman melanoma line cDNA library constructed in the λZAP vector (Stratagene). Library screening was performed using techniques well-known in the art as described in Bunzow et al. (1988, Nature 336: 783-787) at moderate stringency (40% formamide, 1M NaCl, 50mM Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, 0.2% sodium dodecyl sulfate, 100μg/ml salmon sperm DNA, 10X Denhardt's solution). One cDNA clone was identified (termed mmelA) and its 2.6 kb cDNA insert was isolated and subcloned into pBKS (Stratagene); the resulting plasmid was called pmmelA. Nucleotide sequence analysis and homology comparisons were done on the OHSU computer system with software provided by Intelligenetics Inc. (Mountain View, CA).

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The nucleotide sequence of pmmelA (the cDNA clone isolated as described above) is shown in Figure 1A (SEQ ID NO:3). The longest open reading frame of this cDNA encodes a predicted protein product of 315 amino acids with a calculated molecular weight of 35.3 kilodaltons (kD). The deduced amino acid sequence is shown in Figure 2 (SEQ ID NO:4) as mouse MSH-R. Single letter amino acid codes are used [see, G. Zubay, Biochemistry (2d ed.), 1988 (MacMillen Publishing: New York) p.33]. Uppercase lettering indicates amino acid residues in common between the receptor proteins shown; lowercase lettering indicates divergent residues.

Hydrophobicity analysis (Kyte & Doolittle, 1982, J. Mol. Biol. <u>157</u>: 105-132) of the deduced amino acid sequence showed that the protein contains seven hydrophobic stretches of 21 to 26 amino acids apiece. Putative transmembrane domains are overlined and designated with Roman numerals.

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### **EXAMPLE 3**

# Construction of Mouse αMSH<sup>R</sup> Expression Plasmids, DNA Transfection and Functional Expression of the αMSH<sup>R</sup> Gene Product

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In order to biochemically characterize the putative mouse  $\alpha MSH^R$  cDNA isolated as in Example 2, and to confirm that it encodes an  $\alpha MSH$  receptor, mmelA was cloned into a mammalian expression vector, this vector transfected into human 293 cells, and cell lines generated that expressed the putative  $\alpha MSH^R$  receptor at the cell surface. Such cells and membranes isolated from such cells were used for biochemical characterization experiments described below.

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The entire coding region of the  $\alpha$ MSH<sup>R</sup> cDNA insert from mmelA contained in a 2.1kb fragment was excised from pBSK and subcloned into the *BamHI/XhoI* sites of pcDNAI/neo expression vector (Invitrogen, San Diego, CA). The resulting plasmid was called pcDNA-mmelA. pcDNA-mmelA plasmid DNA was prepared in large-scale through one cycle of CsCl gradient ultracentrifugation and 20  $\mu$ g pcDNA-mmelA DNA were transfected into each 100mm dish of 293 cells using the calcium phosphate method (*see* Chen & Okayama, 1987, Mol. Cell. Biol. 7: 2745-2752). After transfection, cells were cultured in DMEM media supplemented with 10% calf serum in a 3% CO<sub>2</sub> atmosphere at 37°C. Selection was performed with neomycin (G418; GIBCO) at a concentration of 1000  $\mu$ g/ml; selection was started 72 hr after transfection and continued for 3 weeks.

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The αMSH<sup>R</sup> is known to couple to G-proteins and thereby activate adenyl cyclase, increasing intracellular levels of cAMP (see Buckley & Ramachandran, 1981, Proc. Natl. Acad. Sci. USA 78: 7431-7435; Grahame-Smith et al., 1967, J. Biol. Chem 242: 5535-5541; Mertz & Catt, 1991, Proc. Natl. Acad. Sci. USA 88: 8525-8529; Pawalek et al., 1976, Invest. Dermatol. 66: 200-209). This

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property of cells expressing the  $\alpha$ MSH receptor was used analyze expression of the  $\alpha$ MSH receptor in cell colonies transfected with the expression vectors described herein as follows. Cells ( $\sim 1 \times 10^6$ ) were plated in 6-well dishes, washed once with DMEM containing 1% bovine serum albumin (BSA) and 0.5mM IBMX (a phosphodiesterase inhibitor), then incubated for 45 minutes at 37°C with varying concentrations of the melanotropic peptides  $\alpha$ MSH,  $\beta$ MSH,  $\gamma$ MSH, the MSH peptide analogues Nle<sup>4</sup>, D-Phe<sup>7</sup>- $\alpha$ MSH (NDP-MSH), and ACTH. Following hormone treatment, the cells were washed twice with phosphate buffered saline and intracellular cAMP extracted by lysing the cells with 1ml of 60% ethanol. Intracellular cAMP concentrations were determined using an assay (Amersham) which measures the ability of cAMP to displace [8-³H] cAMP from a high affinity cAMP binding protein (see Gilman, 1970, Proc. Natl. Acad. Sci. USA 67: 305-312).

The results of these experiments are shown in Figure 3. The abscissa indicates the concentration of each hormone and the ordinate indicates the percentage of basal intracellular cAMP concentration achieved by each treatment. Points indicate the mean of duplicate incubations; the standard error did not exceed 15% for any data point. None of the peptides tested induced any change in intracellular cAMP in cells containing the vector alone. Cells expressing the murine  $\alpha$ MSH receptor responded to melanotropic peptides with a 2-3 fold elevation of intracellular cAMP, similar to levels of cAMP induced by these peptides in the Cloudman cell line (see Pawalek, 1985, Yale J. Biol. Med. 58: 571-578). The EC<sub>50</sub> values determined for  $\alpha$ MSH (2.0x10<sup>-9</sup>M), ACTH (8.0x10<sup>-9</sup>M) and the superpotent MSH analogue NDP-MSH (2.8x10<sup>-11</sup>M) correspond closely to reported values (see Tatro et al., 1990, Cancer Res. 50: 1237-1242). As expected, the  $\beta$ MSH peptide had an EC<sub>50</sub> value comparable to  $\alpha$ MSH<sup>22</sup> while  $\gamma$ MSH had little or no activity (see Slominski et al., 1992, Life Sci. 50: 1103-1108), confirming the identity of this receptor as a melanocyte  $\alpha$ MSH receptor.

### **EXAMPLE 4**

# Isolation and Characterization of a Human aMSHR Genomic Clone

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In order to isolate a human counterpart of the murine melanocyte  $\alpha$ MSH receptor gene, a human genomic library was screened at high stringency (50% formamide, 42°C) using the human PCR fragments isolated as described in Example 1. Two different types of sequences were isolated, corresponding to the two PCR fragments, and were found to encode highly related G protein-coupled receptors. These genomic clones were sequenced as described in Example 2. One of these genomic clones was determined to encode an human MSH receptor (SEQ ID NO:5). The human MSH receptor has a predicted amino acid sequence (SEQ ID NO:6) that is 75% identical and colinear with the mouse  $\alpha$ MSH receptor cDNA sequence (Figure 2), represented as human MSH-R. The predicted molecular weight of the human MSH<sup>R</sup> is 34.7kD.

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The predicted amino acid sequences of the mouse  $\alpha MSH^R$  (SEQ ID NO:4) and human MSHR (SEQ ID NO:6) are aligned in Figure 2. These sequences define the melanocortin receptors as a novel subfamily of the G protein-coupled receptors with a number of unusual features. The melanocortin receptors are the smallest G protein-coupled receptors identified to date (297-317aa) resulting from a short amino terminal extracellular domain, a short carboxy-terminal intracellular domain, and a very small third intracellular loop. The melanocortin receptors are lack several amino acid residues present in most G protein coupled receptors (see Probst et al., 1992, DNA & Cell Biol. 11: 1-20), including the proline residues in the 4th and 5th transmembrane domains, likely to introduce a bend in the alpha helical structure of the transmembrane domains and thought to be involved in the formation of the binding pocket (see Applebury & Hargrave, 1986, Vision Res. 26: 1881-1895), and one or both of the cysteine residues thought to form a disulfide bond between the first and second extracellular loops (see Dixon et al., 1987, EMBO J. 6: 3269-3275 and Karnik et al., 1988, Proc. Natl. Acad. Sci. USA 85: 8459-8463). Remarkably, the melanocortin receptors do not appear highly related to the other G protein-coupled receptors which recognize peptide ligands, such as the receptors for bombesin (see Spindel et al., 1990, Mol.

Endocrinol. 4: 1956-1963) or substance K (see Masu et al., 1987, Nature 329: 836-838) but rather, are more closely related to the receptor for  $\Delta^9$ -tetradhydrocannabinol (see Matsuda et al., 1990, Nature 346: 561-564). The cannabinoid receptor also lacks the conserved proline in transmembrane 5 and the cysteine in the first extracellular loop necessary for disulfide bond formation. Least parsimony analysis with the receptor sequences shown in Figure 2 suggests the cannabinoid and melanocortin receptors may be evolutionarily related and form a subfamily distinct from the peptide receptors and the amine receptors. Regardless of whether the similarities are the result of evolutionary conservation or convergence, the sequence and putative structural similarities between the melanocortin and cannabinoid receptors may be informative in the search for the endogenous cannabinoid-like ligand.

**EXAMPLE 5** 

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## Tissue Distribution of αMSH Receptors

To further gain insight into these receptors, we have examined the tissue distribution of their corresponding mRNAs from various tissues by performing Northern hybridization experiments on RNA isolated from various tissues (see Maniatis et al., ibid.). The results of these experiments are shown in Figure 4.

A panel of tissue samples was examined by Northern hybridization analysis performed under high stringency conditions. The same nitrocellulose filter was hybridized successively with a human MSH receptor probe and a mouse MSH receptor probe to determine the distribution of each receptor mRNA. The murine MSH receptor is encoded predominantly by a single mRNA species of 3.9kb, while the human MSH receptor is encoded, in two melanoma samples, predominantly by a 3.0kb species. High levels of receptor mRNA are seen in both primary mouse melanocytes and mouse melanoma cell lines. In contrast, extremely low levels of receptor mRNA were detected in primary human melanocytes, and many human melanoma samples (see melanoma 1, Fig. 4). Most intriguing is the dramatic elevation of MSH-R mRNA seen thus far in 3 of 11 samples tested, such as is seen in melanoma sample #2 (Fig. 4).

Additionally, we have been unable to detect expression in the brain of any of the receptors described here, despite extensive documentation of MSH binding sites there as well as in other tissues. These finding suggest the existence of alternate forms of these or related receptors that may be specifically expressed in brain tissue.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

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### SEQUENCE LISTING

- (1) GENERAL INFORMATION:
  - (i) APPLICANT: Cone, Roger D

    Mountjoy, Kathleen G
  - (ii) TITLE OF INVENTION: Melanocyte Stimulating Hormone Receptor and Uses
  - (iii) NUMBER OF SEQUENCES: 6
  - (iv) CORRESPONDENCE ADDRESS:
    - (A) ADDRESSEE: Allegretti & Witcoff, Ltd.
    - (B) STREET: 10 South Wacker Drive, Suite 3000
    - (C) CITY: Chicago
    - (D) STATE: Illinois
    - (E) . COUNTRY: USA
    - (F) ZIP: 60606
  - (v) COMPUTER READABLE FORM:
    - (A) MEDIUM TYPE: Floppy disk
    - (B) COMPUTER: IBM PC compatible
    - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
    - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
  - (vi) CURRENT APPLICATION DATA:
    - (A) APPLICATION NUMBER: PCT/US93/03247
    - (B) FILING DATE: 07-APR-1993
    - (C) CLASSIFICATION:
  - (viii) ATTORNEY/AGENT INFORMATION:
    - (A) NAME: Noonan, Kevin E
    - (B) REGISTRATION NUMBER: 35,303
    - (C) REFERENCE/DOCKET NUMBER: 92,154-A
    - (ix) TELECOMMUNICATION INFORMATION:
      - (A) TELEPHONE: 312-715-1000
      - (B) TELEFAX: 312-715-1234
      - (C) TELEX: 910-221-5317
- (2) INFORMATION FOR SEQ ID NO:1:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 33 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: DNA (genomic)
  - (ix) FEATURE:
    - (A) NAME/KEY: misc feature

(B)	LOCATION:	1.	.33
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- (D) OTHER INFORMATION: /function= "Degenerate oligonucleotide primer (sense)"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

### GAGTCGACCT GTGYGYSATY RCTKGACMGS TAC

33

- (2) INFORMATION FOR SEQ ID NO:2:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 31 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: DNA (genomic)
  - (ix) FEATURE:
    - (A) NAME/KEY: misc\_feature
    - (B) LOCATION: 1..31
    - (D) OTHER INFORMATION: /function= "Degenerate oligonucleotide primer (antisense)"
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

### CAGAATTCAG WAGGGCACCA GCAGASRYGA A

- (2) INFORMATION FOR SEQ ID NO:3:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 1260 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
    - (ii) MOLECULE TYPE: CDNA
    - (ix) FEATURE:
      - (A) NAME/KEY: CDS
      - (B) LOCATION: 15..959
    - (ix) FEATURE:
      - (A) NAME/KEY: 5'UTR
      - (B) LOCATION: 1..14
    - (ix) FEATURE:
      - (A) NAME/KEY: 3'UTR
      - (B) LOCATION: 960..1260

Asn	Ala	Thr	Ser 20	His	Leu	Gly	Leu	Ala 25	Thr	Asn	Gln	Ser	Glu 30	Pro	Tr
Сув	Leu	Tyr 35	Val	Ser	Ile	Pro	<b>А</b> вр 40	Gly	Leu	Phe	Leu	Ser 45	Leu	Gly	Let
Val	Ser 50	Leu	Val	Glu	Asn	Val 55	Leu	Val	Val	Ile	Ala 60	Ile	Thr	Lys	Ası
Arg 65	Asn	Leu	His	Ser	Pro 70	Met	Tyr	Tyr	Phe	Ile 75	Сув	Cys	Leu	Ala	Let 80
Ser	Asp	Leu	Met	Val 85	Ser	Val	Ser	Ile	<b>Val</b> 90	Leu	Glu	Thr	Thr	Ile 95	Ile
Leu	Leu	Leu	Glu 100	Val	Gly	Ile	Leu	Val 105	Ala	Arg	Val	Ala	Leu 110	Val	Gli
		115					120				Gly	125			
	130					135					Arg 140				
145					150					155	Leu				160
				165					170		Val	*		175	
			180					185			Leu		190		
		195					200				Leu	205			
Phe	Thr 210	Arg	Ala	Cys	Gln	His 215	Val	Gln	Gly	Ile	Ala 220	Gln	Leu	His	Lys
Arg 225	Arg	Arg	Ser	Ile	Arg 230	Gln	Gly	Phe	Сув	Leu 235	Lys	Gly	Ala	Ala	Thr 240
Leu	Thr	Ile	Leu	Leu 245	Gly	Ile	Phe	Phe	Leu 250	Сув	Trp	Gly	Pro	Phe 255	Phe
Leu	His	Leu	Leu 260	Leu	Ile	Val	Leu	Сув 265	Pro	Gln	His	Pro	Thr 270	СЛа	Ser
Cys	Ile	Phe 275	Lys	Asn	Phe	Asn	Leu 280	Phe	Leu	Leu	Leu	Ile 285	Val	Leu	Ser
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Met	Thr	Leu	Lys	Glu	Val	Leu	Leu	Сув	Ser	Trp
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- (2) INFORMATION FOR SEQ ID NO:5:
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    - (A) LENGTH: 1633 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: cDNA
  - (ix) FEATURE:
    - (A) NAME/KEY: CDS
    - (B) LOCATION: 462..1415
  - (ix) FEATURE:
    - (A) NAME/KEY: 5'UTR
    - (B) LOCATION: 1..461
  - (ix) FEATURE:
    - (A) NAME/KEY: 3'UTR
    - (B) LOCATION: 1416..1633
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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AGGACGGTCC AGAGGTGTCG AAATGTCCTG GGAACCTGAG CAGCAGCCAC CAGGGAAGAG	180
GCAGGGAGGG AGCTGAGGAC CAGGCTTGGT TGTGAGAATC CCTGAGCCCA GGCGGTTGAT	240
GCCAGGAGGT GTCTGGACTG GCTGGGCCAT GCCTGGGCTG ACCTGTCCAG CCAGGGAGAG	300
GGTGTGAGGG CAGATCTGGG GGTGCCCAGA TGGAAGGAGG CAGGCATGGG GACACCCAAG	360
GCCCCCTGGC AGCACCATGA ACTAAGCAGG ACACCTGGAG GGGAAGAACT GTGGGGACCT	420
GGAGGCCTCC AACGACTCCT TCCTGCTTCC TGGACAGGAC T ATG GCT GTG CAG  Met Ala Val Gln  1	473
GGA TCC CAG AGA AGA CTT CTG GGC TCC CTC AAC TCC ACC CCC ACA GCC Gly Ser Gln Arg Arg Leu Leu Gly Ser Leu Asn Ser Thr Pro Thr Ala 5 10 15 20	521
ATC CCC CAG CTG GGG CTG GCT GCC AAC CAG ACA GGA GCC CGG TGC CTG  Ile Pro Gln Leu Gly Leu Ala Ala Asn Gln Thr Gly Ala Arg Cys Leu  25 30 35	569

GAG	GTG	TCC	ATC	TCT	GAC	GGG	CTC	TTC	CTC	AGC	CTG	GGG	CTG	GTG	AGC	617
Glu	Val	Ser	Ile 40	Ser	Двр	Gly	Leu	Phe 45	Leu	Ser	Leu	Gly	Leu 50	Val	Ser	
TTG	GTG	GAG	AAC	GCG	CTG	GTG	GTG	GCC	ACC	ATC	GCC	AAG	AAC	CGG	AAC	665
Leu	Val	Glu 55	Asn	Ala	Leu	Val	Val 60	Ala	Thr	Ile	Ala <sub>.</sub>	65	Asn	Arg	Asn	
CTG	CAC	TCA	CCC	ATG	TAC	TGC	TTC	ATC	TGC	TGC	CTG	GCC	TTG	TCG	GAC	713
Leu	His 70	Ser	Pro	Met	Tyr	Сув 75	Phe	Ile <sup>.</sup>	Сўв	Cys	Leu 80	Ala	Leu	Ser	Asp	
CTG	CTG	GTG	AGC	GGG	ACG	AAC	GTG	CTG	GAG	ACG	GCC	GTC	ATC	CTC	CTG	761
Leu 85	Leu	Val	Ser	Gly	Thr 90	Asn	Val	Leu	Glu	Thr 95	Ala	Val	Ile	Leu	Leu 100	
CTG	GAG	GCC	GGT	GCA	CTG	GTG	GCC	CGG	GCT	GCG	GTG	CTG	CAG	CAG	CTG	809
					Leu											
GAC	AAT	GTC	ATT	GAC	GTG	ATC	ACC	TGC	AGC	TCC	ATG	CTG	TCC	AGC	CTC	857
Asp	Asn	Val	11e 120	Asp	Val	Ile	Thr	Сув 125	Ser	Ser	Met	Leu	Ser 130	Ser	Leu	
TGC	TTC	CTG	GGC	GCC	ATC	GCC	GTG	GAC	CGC	TAC	ATC	TCC	ATC	TTC	TAC	905
Сув	Phe	Leu 135	Gly	Ala	Ile	Ala	Val 140	Asp	Arg	Tyr	Ile	Ser 145	Ile	Phe	Tyr	
GCA	CTG	CGC	TAC	CAC	AGC	ATC	GTG	ACC	CTG	CCG	CGG	GCG	CCG	CGA	GCC	953
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Val 165	Ala	Ala	Ile	Trp	Val 170	Ala	Ser	Val	Val	Phe 175	Ser	Thr	Leu	Phe	Ile 180	
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Ala	Tyr	Tyr	Asp	His 185	Val	Ala	Val	Leu	Leu 190	Сув	Leu	Val	Val	Phe 195	Phe	
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					GGC											1 193
Arg	Pro 230	Val	His	Gln	Gly	Phe 235		Leu	Lys	Gly	Ala 240	Val	Thr	Leu	Thr	

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CTC Leu	ACA Thr	CTC Leu	ATC Ile	GTC Val 265	CTC Leu	TGC Cys	CCC Pro	GAG Glu	CAC His 270	CCC Pro	ACG Thr	TGC Cys	GGC Gly	TGC Cys 275	ATC Ile	1289
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- (2) INFORMATION FOR SEQ ID NO:6:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 317 amino acids
    - (B) TYPE: amino acid
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: protein
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Ala Arg Cys Leu Glu Val Ser Ile Ser Asp Gly Leu Phe Leu Ser Leu 35 40 45

Gly Leu Val Ser Leu Val Glu Asn Ala Leu Val Val Ala Thr Ile Ala
50 55 60

Lys Asn Arg Asn Leu His Ser Pro Met Tyr Cys Phe Ile Cys Cys Leu 65 70 75 80

Ala	Leu	Ser	Asp	Leu 85	Leu	Val	Ser	Gly	Thr 90	Asn	Val	Leu	Glu	Thr 95	Ala
Val	Ile	Leu	Leu 100	Leu	Glu	Ala	Gly	Ala 105	Leu	Val	Ala	Arg	Ala 110	Ala	Val
Leu	Gln	Gln 115	Leu	Asp	Asn	Val	Ile 120	Asp	Val	Ile	Thr	Сув 125	Ser	Ser	Met
Leu	Ser 130	Ser	Leu	Сув	Phe	Leu 135	Gly	Ala	Ile	Ala	Val 140	Asp	Arg	Tyr	Ile
Ser 145	Ile	Phe	Tyr	Ala	Leu 150	Arg	Tyr	His	Ser	Ile 155	<b>V</b> al	Thr	Leu	Pro	Arg 160
Ala	Pro	Arg	Ala	Val 165	Ala	Ala	Ile	Trp	Val 170	Ala	Ser	Val	Val	Phe 175	Ser
Thr	Leu	Phe	Ile 180	Ala	Tyr	Tyr	Asp	His 185	Val	Ala	Val	Leu	Leu 190	Сув	Leu
Val	Val	Phe 195	Phe	Leu	Ala	Met	Leu 200	Val	Leu	Met	Ala	Val 205	Leu	Tyr	Val
His	Met 210	Leu	Ala	Arg	Ala	Сув 215	Gln	His	Ala	Gln	Gly 220	Ile	Ala	Arg	Leu
His 225	ГÀВ	Arg	Gln	Arg	Pro 230	Val	His	Gln	Gly	Phe 235	Gly	Leu	Lys	Gly	Ala 240
Val	Thr	Leu	Thr	11e 245	Leu	Leu	Gly	Ile	Phe 250	Phe	Leu	Сув	Trp	Gly 255	Pro
Phe	Phe	Leu	Нів 260	Leu	Thr	Leu	Ile	Val 265	Leu	Cys	Pro	Glu	His 270	Pro	Thr
Сув	Gly	Сув 275	Ile	Phe	Lys	Asq	Phe 280	Asn	Leu	Phe	Leu	Ala 285	Leu	Ile	Ile
Сув	Asn 290	Ala	Ile	Ile	Asp	Pro 295	Leu	Ile	Tyr	Ala	Phe 300	His	Ser	Gln	Glu
Leu 305	Arg	Arg	Thr	Leu	Lys 310	Glu	Val	Leu	Thr	Сув 315	Ser	Trp			

### WHAT WE CLAIM IS:

- l. A nucleic acid comprising a nucleotide sequence encoding a mammalian melanocyte stimulating hormone receptor.
- 2. A nucleic acid according to Claim I wherein the mammalian melanocyte stimulating hormone receptor is the mouse melanocyte stimulating hormone receptor.
- 3. A nucleic acid according to Claim I wherein the nucleotide sequence is substantially homologous to the sequence in Figure 1A (SEQ ID NO:3).
- 4. A nucleic acid according to Claim I wherein the mammalian melanocyte stimulating hormone receptor is the human melanocyte stimulating hormone receptor.
- 105. A nucleic acid according to Claim I wherein the nucleotide sequence is substantially homologous to the sequence in Figure IB (SEQ ID NO:5).
- 6. A DNA sequence according to Claim I wherein the mammalian melanocyte stimulating hormone receptor encoded therein has the melanotropic peptide response properties described in Figure 3.
- 157. A homogeneous composition of a 35.3 kilodalton melanocyte stimulating hormone receptor or derivative thereof, wherein the amino acid sequence of the melanocyte stimulating hormone receptor or derivative thereof comprises the mouse MSH-R sequence shown in Figure 2 (SEQ ID NO:4).
- 8. A homogeneous composition of a 34.6 kilodalton melanocyte stimulating horm20 receptor or derivative thereof, wherein the amino acid sequence of the melanocyte stimulating hormone receptor or derivative thereof comprises the human MSH-R sequence shown in Figure 2 (SEQ ID NO:6).
- 9. A nucleic acid hybridization probe for the detection of mammalian melanocyte stimulating hormone receptor expression comprising the nucleotide sequence of Claim 3.
- 10. The nucleic acid hybridization probe according to Claim 9 whereby the probe is adapted for use in the detection and diagnosis of genetic disease in a human.
- 11. The nucleic acid hybridization probe according to Claim 9 whereby the probablis adapted for use in the detection, isolation and characterization of novel mammalian receptor genes.
- 12. A nucleic acid hybridization probe for the detection of mammalian melanocyte stimulating hormone receptor expression comprising the nucleotide sequence of Claim 5.
- 35 l3. The nucleic acid hybridization probe according to Claim 12 whereby the probe is adapted for use in the detection and diagnosis of genetic disease in a

human.

- 14. The nucleic acid hybridization probe according to Claim 12 whereby the probe is adapted for use in the detection, isolation and characterization of novel mammalian receptor genes.
- 515. A recombinant expression construct comprising a nucleotide sequence encoding a mammalian melanocyte stimulating hormone receptor.
- 16. A recombinant expression construct comprising the DNA sequence of Claim 3, wherein the construct is capable of expressing the mouse melanocyte stimulating hormone receptor in a transformed eukaryotic cell culture.
- 1017. A recombinant expression construct comprising the DNA sequence of Claim 5, wherein the construct is capable of expressing the human melanocyte stimulating hormone receptor in a transformed eukaryotic cell culture.
- 18. The recombinant expression construct of Claim 15 comprising pcDNAI/neo sequences.
- 15 19. A eukaryotic cell culture transformed with the expression construct of Claim 16, wherein the transformed eukaryotic cell culture is capable of expressing mouse melanocyte stimulating hormone receptor.
- 20. A eukaryotic cell culture transformed with the expression construct of Claim 17, wherein the transformed eukaryotic cell culture is capable of expressing the Maman melanocyte stimulating hormone receptor.
- 21. A method of screening a compound as an inhibitor of agonist binding to a mammalian melanocyte stimulating hormone receptor, the method comprising the following steps:
  - (a) transforming a eukaryotic cell culture with an expression construct as
  - 25 in Claim 15 capable of expressing the melanocyte stimulating hormone receptor in a eukaryotic cell; and
    - (b) assaying for ability of the compound to inhibit the binding of a detectable melanocyte stimulating hormone receptor agonist.
- 22. The method of Claim 21 wherein the mammalian melanocyte stimulating horma@ae receptor is the mouse melanocyte stimulating hormone receptor.
- 23. The method of Claim 21 wherein the mammalian melanocyte stimulating hormone receptor is the human melanocyte stimulating hormone receptor.
- 24. A method of quantitatively detecting a compound as an inhibitor of agonist binding to a mammalian melanocyte stimulating hormone receptor, the method compassing the following steps:
  - (a) transforming a eukaryotic cell culture with an expression construct as

- in Claim 15 capable of expressing the mammalian melanocyte stimulating hormone receptor in a sukaryotic cell; and
- (b) assaying for amount of a compound by measuring the extent of inhibition of binding of a detectable receptor agonist.
- 525. The method of Claim 24 wherein the mammalian melanocyte stimulating hormone receptor is the mouse melanocyte stimulating hormone receptor.
- 26. The method of Claim 24 wherein the mammalian melanocyte stimulating hormone receptor is the human melanocyte stimulating hormone receptor.
- 27. The method of Claim 24 wherein the compound to be tested is present in a human.
  - 28. The method of Claim 24 wherein the compound is present in human blood.
- 29. The method of Claim 24 wherein the compound is present in human cerebrospinal fluid.
  - 30. The method of Claim 24 wherein the compound is unknown.
- 1531. An antibody or fragment thereof that is immunologically reactive to a mammalian melanocyte stimulating hormone receptor.
- 32. The antibody according to Claim 31, wherein the antibody is a monoclonal antibody.
- 33. The antibody according to Claim 31, wherein the mammalian melanocyte stim20ating hormone receptor is the mouse melanocyte stimulating hormone receptor.
- 34. The antibody according to Claim 31, wherein the mammalian melanocyte stimulating hormone receptor is the human melanocyte stimulating hormone receptor.
- 35. A cell line which produces an antibody or fragment thereof that is immunologically reactive to a mammalian melanocyte stimulating hormone receptor.
- 2536. The cell line according to Claim 35, wherein the antibody is a monoclonal antibody.
- 37. The cell line according to Claim 35, wherein the mammalian melanocyte stimulating hormone receptor is the mouse melanocyte stimulating hormone receptor.
- 38. The cell line according to Claim 35, wherein the mammalian melanocyte stimmalating hormone receptor is the human melanocyte stimulating hormone receptor.
- 39. A pharmaceutical composition comprising a therapeutically effective amount of an antibody or fragment thereof according to claim 31 in a pharmaceutically acceptable carrier.
- 40. An epitope of a mammalian melanocyte stimulating hormone receptor wherain the epitope is immunologically reactive to the antibody or fragment thereof according to claim 31.

- 41. The epitope according to claim 40 wherein the mammalian melanocyte stimulating hormone receptor is the mouse melanocyte stimulating hormone receptor.
- 42. The epitope according to claim 40 wherein the mammalian melanocyte stimulating hormone receptor is the human melanocyte stimulating hormone receptor.
- 543. A chimeric antibody that is immunologically reactive to a mammalian melanocyte stimulating hormone receptor.
- 44. The chimeric antibody according to claim 43 wherein the mammalian melanocyte stimulating hormone receptor is the mouse melanocyte stimulating hormone receptor.
- 1045. The chimeric antibody according to claim 43 wherein the mammalian melanocyte stimulating hormone receptor is the human melanocyte stimulating hormone receptor.

### Figure 1A

10 20 30	.0 50 60 70
TTCCTGACAA GACTATGTCC ACTCAGGAGC CCCAC	AAGAG TCTTCTGGGT TCTCTCAACT CCAATGCCAC
80 90 100 11	0 120 130 140
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150 160 170 18	0 190 200 210
TICCTCAGCC TAGGGCTGGT GAGTCTGGTG GAGAA	TOTGC TGGTTGTGAT AGCCATCACC AAAAACCGCA
220 230 240 25	200
ACCTGCACTC GCCCATGTAT TACTTCATCT GCTGC	D 260 270 280 CTGGC CCTGTCTGAC CTGATGGTAA GTGTCAGCAT
CGTGCTGGAG ACTACTATCA TCCTCCTCCT CCACC	330 340 350
	GGGC ATCCTGGTGG CCAGAGTGGC TTTGGTGCAG
360 370 380 390	400 410 420
CAGCTGGACA ACCTCATTGA CGTGCTCATC TGTGGG	TCCA TGGTGTCCAG TCTCTGCTTC CTGGGCATCA
430 440 450 466	
TIGCTATAGA CCGCTACATC TCCATCTTCT ATGCGC	TGCG TTATCACAGC ATCCTCACCC TCCCCACAG
500 510 520 530 ACGACGGCCT GTCGTGGGCA TCTGGATGGT CAGCAT	540 550 560
570 580 590 600	610 620 630
CACACAGCCG TTCTGCTCTG CCTCGTCACT TTCTTT	CTAG CCATGCTGGC ACTCATGGCG ATTCTGTATG
640 650 660 670	
CCCACATGTT CACGAGAGCG TGCCAGCACG TCCAGG	GCAT TGCCCAGCTC CACAAAACGC CCCCCTCCAT
CCGCCAAGGC TTCTGCCTCA AGGGTGCTGC CACCCT	750 760 770
	ACCITATION GGATTITCTT CCTGTGCTGG
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GGCCCCTTCT TCCTGCATCT CTTGCTCATC GTCCTC	GCC CTCAGCACCC CACCTGCAGC TGCATCTTCA
850 860 870 RRA	000
AGAACTICAA CCTCTTCCTC CTCCTCATCG TCCTCAC	SCTC CACTGTTGAC CCCCTCATCT ATCCTTTGGG
920 930 940 950 CAGCCAGGAG CTCCGCATGA CACTCAACGA CCTCCGCATGA	960 970 980
CAGCCAGGAG CTCCGCATGA CACTCAAGGA GGTGCTC	
990 1000 1010 1020	1030 1040 1050
AGGGTGACAG TGATATCCAG TGGCCTGCAT CTGTGAG	ACC ACAGGTACTC ATCCCTTCCT GATCTCCATT
TGTCTAAGGG TCGACAGGAT GAGCTTTAAA ATAGAAA	1100 1110 1120
1130 1140 1150 1160	1170 1180 1190
TGTGACTGCA GGGCTCACCC AGGGCAGCTA CGGGAAG	TGG AGGAGACAGG GATGGGAACT CTAGCCCTGA
1200 1210 1220 1230	12/0
GCAAGGGTCA GACCACAGGC TCCTGAAGAG CTTCACC	1240 1250 1260 ICT CCCCACCTAC AGGGAACTCC TECTGAAGGG

## Figure 1B

10	20	30 ATGGAGGGCT	40	50	60	70
80		100		120		140
		CCTGGCCCAG				
150 AAATGTCCTG	160 GGAACCTGAG	170 CAGCAGCCAC			200 AGCTGAGGAC	210 CAGGCTTGGT
220 TGTGAGAATC		240 GGCGGTTGAT			270 GCTGGGCCAT	280 GCCTGGGCTG
290 ACCTGTCCAG		310 GGTGTGAGGG				350 CAGGCATGGG
360 GACACCCAAG	370 GCCCCCTGGC	380 AGCACCATGA	390 ACTAAGCAGG		410 GGGAAGAACT	420 GTGGGGACCT
		450 TCCTGCTTCC				490 AGAGAAGACT
500 TCTGGGCTCC	510 CTCAACTCCA	520 CCCCCACAGC	530 CATCCCCCAG	540 CTGGGGCTGG	550 CTGCCAACCA	560 GACAGGAGCC
570 CGGTGCCTGG	580 AGGTCTCCAT	590 CTCTGACGGG	600 CTCTTCCTCA	610 GCCTGGGGCT	620 GGTGAGCTTG	630 GTGGAGAACG
640 CGCTGGTGGT	650 GGCCACCATC	660 GCCAAGAACC	670 GGAACCTGCA	680 CTCACCCATG	690 TACTGCTTCA	700 TCTGCTGCCT
		730 TGAGCGGGAC			760 TCATCCTCCT	770 GCTGGAGGCC
780 GGTGCACTGG		- 800 TGCGGTGCTG	810 CAGCAGCTGG	820 ACAATGTCAT	830 TGACGTGATC	840 ACCTGCAGCT
850 CCATGCTCTC	860 CAGCCTCTGC	870 TTCCTGGGCG		890 GGACCGCTAC	900 ATCTCCATCT	910 TCTACGCACT
920 GCGCTACCAC	930 AGCATCGTGA	940 CCCTGCCGCG	950 GGCGCCGCGA			980 GGCCAGTGTC
990 GTCTTCAGCA	1000 CGCTCTTCAT	1010 CGCCTACTAC	1020 GACCACGTGG	1030 CCGTCCTGCT	1040 GTGCCTCGTG	LOSO GTCTTCTTCC
1060 TGGCTATGCT		1080 GCCGTGCTGT				1120 ACGCCCAGGG
1130 CATCGCCCGG	1140 CTCCACAAGA	1150 GGCAGCGCCC	1160 GGTCCACCAG	1170 GGCTTTGGCC	1180 TTAAAGGCGC	1190 TGTCACCCTC
1200 ACCATCCTGC		1220 CTTCCTCTGC	1230 TGGGGCCCCT			1260 ATCGTCCTCT

## Figure 1C

1270	1280	1290	1300	1310		1330
GCCCCGAGCA	CCCCACGTGC	GGCTGCATCT	TCAAGAACTT	CAACCTCTTT	CTCGCCCTCA	TCATCTGCAA
1340	1350		1370	1380	1390	1400
TGCCATCATC	GACCCCCTCA	TCTACGCCTT	CCACAGCCAG	GAGCTCCGCA	GGACGCTCAA	GGAGGTGCTG
1410	1420		1440		1460	1470
ACATGCTCCT	GCTGAGCGCG	GTGCACGCGC	TTTAAGTGTG	CTGGGCAGAG	GGAGGTGGTG	ATATTGTGGT
1480	1490	1500	1510	1520		1540
CIGGITCCIG	TGTGACCCTG	GGCAGTTCCT	TACCTCCCTG	GTCCCCGTTT	GTCAAAGAGG	ATGGACTAAA
1550	1560	1570	1580	1590	1600	1610
TGATCTCTGA	AAGTGTTGAA	GCGCGGACCC	TTCTGGGCAG	GGAGGGGTCC	TGCAAAACTC	CAGGCAGGAC
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1620	1630					
TTCTCACCAG	CAGTCGTGGG	AAC				

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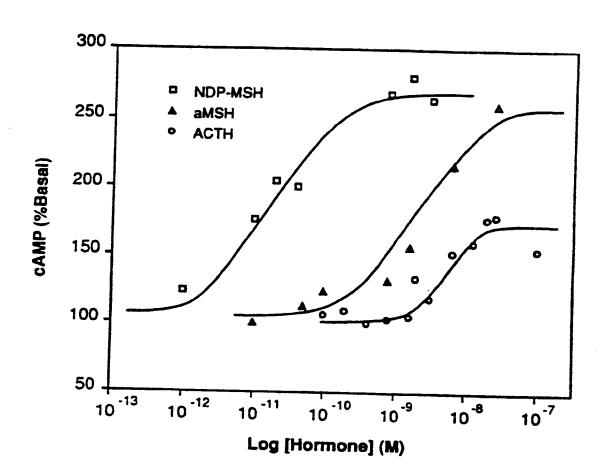
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Figure 2C	mouse MSH-R	human MSH-R	human ACTH-R	rat cannab.	mouse MSH-R	human MSH-R	human ACTH-R	rat cannab.	mouse MSH-R	human MSH-R	human ACTH-R	rat cannab.

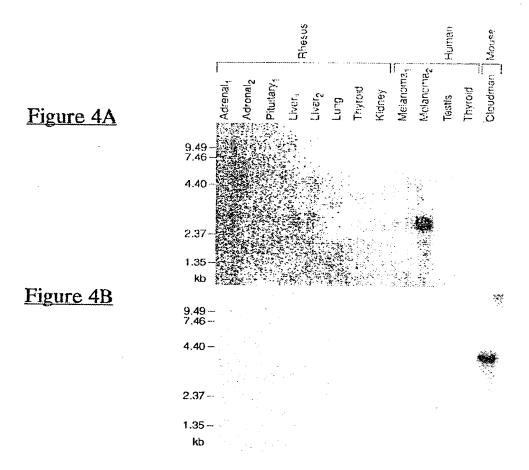
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Figure 2E	mouse MSH-R	human MSH-R	human ACTH-R	rat cannab.		mouse MSH-R	human MSH-R	human ACTH-R	rat cannab.		mouse MSH-R	human MSH-R	human ACTH-R	rat cannab.

Figure 3





## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 93/03247

Date of the Actual Completion of the International Search  Date of Mailing of this International Search Report  15 SEPTEMBER 1993					
Int.Cl. 5 C12N15/12; C07K13/00; C12P21/08; C12N5/10 C12N15/62; A61K37/02; A61K39/395; C12Q1/68  Int.Cl. 5 Milatinum Decomentation Searched  Classification System  Classification Symbols  Int.Cl. 5 C12N ; C07K ; A61K ; G01N  Decomentation Searched other than Minipuum Decomentation to the Extent that rack Decoments are holded in the Fields Searched*  III. DOCUMENTS CONSIDERED TO BE RELEVANT*  Category* Classification Symbols  III. DOCUMENTS CONSIDERED TO BE RELEVANT*  Category* Classification of Decument, 11 with indication, where appropriate, of the relevant passages 12  P, X SCIENCE vol. 257, 28 August 1992, LANCASTER, PA pages 1248 – 1251  Mount, joy Kej Robbins - LS; Mortrud MT; Cone RD; 'The cloning of a family of genes that encode the melanocortin receptors.' see the whole document  P, X FEBS LETTERS. vol. 309, no. 3, 14 September 1992, AMSTERDAM NI, pages 417 – 420  Chhaijaini vivikberg JE 'Molecular cloning and expression of the human melanocyte strinulating hormone receptor cDNA.' see the whole document  *Special categories of died documents: 10  *A document sefficing the general state of the art which is not considered to be of particular relevance the cained severated which as pictural relevance the cained severated which as pictural relevance the claimed severated relevance to the considered to be of particular relevance the claimed severated relevance to the considered to be of particular relevance the claimed severated relevance to the considered to be considered to the considered to the considered to the considered to the considered to be considered to the claimed severated relevance to the considered to be considered to the co	<b>L</b>				
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vol. 309, no. 3, 14 September 1992, AMSTERDAM NL pages 417 - 420 Chhajlani V; Wikberg JE 'Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA.' see the whole document /  *Special categories of cited documents: 10  *A' document defining the general state of the art which is not considered to be of particular referance considered to be of particular referance  *E' earlier document but published on or after the international filing date 1"." document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  "O' document which may throw doubts on priority claim(s) or other special reason (as specified)  "O' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  "O' document referring to an oral disclosure, use, exhibition or other special reason (as specified)  "O' document published after the international filing date but later than the priority of an overally steps when the document is combination being obvious to a person skilled in the art.  "E' document published after the international filing date but later than the priority date claimed invention cannot be considered to involve an inventive step  "Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step  "Y' document published after the international filing date but invention of particular relevance; the claimed invention cannot be considered to involve an inventive step  "Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step  "Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step  "Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step  "Y' document of particular relevance; the claimed invention ca		Mountjoy 'The clo encode t	y KG;Robbins LS;Mortr oning of a family of o the melanocortin rece	genes that	
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III. DOCUMEN	International Application No  NTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)	
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Category	Citation of December 1, 11 and interesting 1, 11 and 11 an	
P,X	THE BIOCHEMICAL JOURNAL vol. 286, 1 September 1992, LONDON, GB pages 377 - 382 Ahmed AR; Olivier GW; Adams G; Erskine ME; Kinsman RG; Branch SK; Moss SH; Notarianni LJ; Pouton CW; 'Isolation and partial purification of a melanocyte-stimulating hormone receptor from B16 murine melanoma cells. A novel approach using a cleavable biotinylated photoactivated ligand and streptavidin-coated magnetic beads.' see the whole document	1-45
<b>A</b>	JOURNAL OF CELLULAR PHYSIOLOGY vol. 137, no. 1, October 1988, WILEY-LISS, INC. pages 35 - 44 Kameyama K; Montague PM; Hearing VJ; 'Expression of melanocyte stimulating hormone receptors correlates with mammalian pigmentation, and can be modulated by interferons.' see the whole document	1-45
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#### PCT

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#### INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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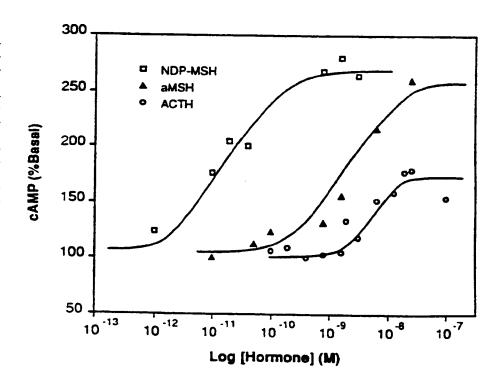
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(54) Title: MAMMALIAN MELANOCYTE STIMULATING HORMONE RECEPTORS AND USES

#### (57) Abstract

The present invention relates to a mammalian melanocyte stimulating hormone receptor. The invention is directed toward the isolation, characterization and pharmacological use of mammalian melanocyte stimulating hormone receptor, the gene corresponding to this receptor, a recombinant eukaryotic expression construct capable of expressing a mammalian melanocyte stimulating hormone receptor in cultures of transformed eukaryotic cells and such cultures of transformed eukaryotic cells that synthesize mammalian melanocyte stimulating hormone receptor. The invention also provides methods for screening MSHR agonists and antagonists in vitro using preparations of receptor from such cultures of eukaryotic cells transformed with a recombinant eukaryotic expression construct comprising the MSHR receptor gene. The invention specifically provides human and mouse MSHR genes.



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## MAMMALIAN MELANOCYTE STIMULATING HORMONE RECEPTORS AND USES

#### **BACKGROUND OF THE INVENTION**

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This invention was made with government support under 1R01DK41921-03, 1R01DK43859-01, and 1P01DK44239-10A1 by the National Institutes of Health. The government has certain rights in the invention.

#### 1. Field of the Invention

This invention relates to melanocyte stimulating hormone receptors from mammalian species and the genes corresponding to such receptors. Specifically, the invention relates to the isolation, cloning and sequencing of a human melanocyte stimulating hormone receptor gene. The invention also relates to the isolation, cloning and sequencing of a mouse melanocyte stimulating hormone The invention relates to the construction of eukaryotic receptor gene. recombinant expression constructs capable of expressing these melanocyte stimulating hormone receptors in cultures of transformed eukaryotic cells, and the production of the melanocyte stimulating hormone receptor in such cultures. The invention relates to the use of such cultures of transformed eukaryotic cells to produce homogeneous compositions of such melanocyte stimulating hormone The invention also provides cultures of such cells producing receptors. melanocyte stimulating hormone receptor for the characterization of novel and useful drugs. Antibodies against and epitopes of these melanocyte stimulating hormone receptor proteins are also provided by the invention.

#### 2. Background of the Invention

The proopiomelanocortin (POMC) gene product is processed to produce a large number of biologically active peptides. Two of these peptides,  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ MSH), and adrenocorticotropic hormone (ACTH) have well understood roles in control of melanocyte and adrenocortical function, respectively. Both of these hormones, however, are found in a variety of forms with unknown functions. The melanocortin peptides also have a diverse

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array of biological activities in other tissues, including the brain, and immune system, and bind to specific receptors there with a distinct pharmacology [see, Hanneman et al., in Peptide Hormone as Prohormones, G. Martinez, ed. (Ellis Horwood Ltd.: Chichester, UK) pp. 53-82; DeWied & Jolles, 1982, Physiol. Rev. 62: 976-1059 for reviews].

A complete understanding of these peptides and their diverse biological activities requires the isolation and characterization of their corresponding receptors. Some biochemical studies have been reported on the prior art.

Shimuze, 1985, Yale J. Biol. Med. <u>58</u>: 561-570 discusses the physiology of melanocyte stimulating hormone.

Tatro & Reichlin, 1987, Endocrinology <u>121</u>: 1900-1907 disclose that MSH receptors are widely distributed in rodent tissues.

Solca et al., 1989, J. Biol. Chem. <u>264</u>: 14277-14280 disclose the molecular weight characterization of mouse and human MSH receptors linked to radioactively and photoaffinity labeled MSH analogues.

Siegrist et al., 1991, J. Receptor Res. 11: 323-331 disclose the quantification of receptors on mouse melanoma tissue by receptor autoradiography.

The present invention comprises a human melanocyte stimulating hormone receptor gene, the nucleotide sequence of this gene and the deduced amino acid sequence of its cognate protein, a homogeneous composition of the melanocyte stimulating hormone receptor, nucleic acid hybridization probes and a method for determining the tissue distribution of expression of the gene, a recombinant expression construct capable of expressing the gene in cultures of transformed eukaryotic cells, and such cultures of transformed eukaryotic cells useful in the characterization of novel and useful drugs. The present invention also comprises the homologue of the human melanocyte stimulating hormone receptor gene from the mouse.

#### **DESCRIPTION OF THE DRAWINGS**

Figure 1 illustrates the nucleotide sequence of the mouse (SEQ ID NO:3) and human (SEQ ID NO:5) melanocyte stimulating hormone receptor.

Figure 2 presents an amino acid sequence comparison between the mouse and human melanocyte stimulating hormone receptor proteins.

Figure 3 illustrates binding of melanocyte stimulating hormone receptor agonists to mouse melanocyte stimulating hormone receptor expressed in human 293 cells.

Figure 4 illustrates the tissue distribution of human (Panel A) and mouse (Panel B) melanocyte stimulating hormone receptor gene expression by Northern blot hybridization.

#### SUMMARY OF THE INVENTION

The present invention relates to the cloning, expression and functional characterization of mammalian melanocyte stimulating hormone receptor (MSH<sup>R</sup>) genes. The invention comprises the nucleotide sequence of these genes encoding the mammalian MSH<sup>R</sup>s and the deduced amino acid sequences of the cognate proteins, as well as tissue distribution patterns of expression of these genes.

In particular, the present invention is directed toward the isolation, characterization and pharmacological use of the human MSH<sup>R</sup>, the gene corresponding to this receptor, a nucleic acid hybridization probe comprising DNA sequences of the human MSH<sup>R</sup>, a recombinant eukaryotic expression construct capable of expressing the human MSH<sup>R</sup> in cultures of transformed eukaryotic cells and such cultures of transformed eukaryotic cells that synthesize the human MSH<sup>R</sup>, a homogeneous composition of the human MSH<sup>R</sup>, and antibodies against and epitopes of the human MSH<sup>R</sup>.

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The present invention is also directed toward the isolation, characterization and pharmacological use of the mouse MSH<sup>R</sup>, the gene corresponding to this receptor, a nucleic acid hybridization probe comprising DNA sequences of the mouse MSH<sup>R</sup>, a recombinant eukaryotic expression construct capable of expressing the mouse MSH<sup>R</sup> in cultures of transformed eukaryotic cells and such cultures of transformed eukaryotic cells that synthesize the mouse MSH<sup>R</sup>, a homogeneous composition of the mouse MSH<sup>R</sup>, and antibodies against and epitopes of the mouse MSH<sup>R</sup>.

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It is an object of the invention to provide a nucleic acid comprising a nucleotide sequence encoding a mammalian MSH<sup>R</sup>. In a preferred embodiment of the invention, the nucleotide sequence encodes the human MSH<sup>R</sup>. In another preferred embodiment, the nucleotide sequence encodes the mouse MSH<sup>R</sup>.

The present invention includes a nucleic acid comprising a nucleotide sequence encoding a human MSH<sup>R</sup> receptor derived from a DNA molecule isolated from a human genomic library (SEQ ID NO:5). In this embodiment of the invention, the nucleotide sequence includes 1635 nucleotides of the human MSH<sup>R</sup> gene comprising 953 nucleotides of coding sequence, 462 nucleotides of

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5' untranslated sequence and 220 nucleotides of 3' untranslated sequence.

The present invention also includes a nucleic acid comprising a nucleotide sequence encoding a mouse MSH<sup>R</sup> derived from a cDNA molecule isolated from a CDNA library constructed with RNA from mouse Cloudman melanoma cells (SEQ ID NO:3). In this embodiment of the invention, the nucleotide sequence includes 1260 nucleotides of the mouse MSH<sup>R</sup> gene comprising 947 nucleotides of coding sequence, 15 nucleotides of 5' untranslated sequence and 298 nucleotides of 3' untranslated sequence.

The invention includes nucleic acids comprising the nucleotide sequences of mammalian MSH<sup>R</sup>s, most preferably mouse and human MSH<sup>R</sup>s (SEQ ID NOs:3&5), and includes allelic variations of these nucleotide sequences and the corresponding MSH<sup>R</sup> molecule, either naturally occurring or the product of *in vitro* chemical or genetic modification, each such variant having essentially the same nucleotide sequence as the nucleotide sequence of the corresponding MSH<sup>R</sup> disclosed herein, wherein the resulting MSH<sup>R</sup> molecule has substantially the same biological properties as the MSH<sup>R</sup> molecule corresponding to the nucleotide sequence described herein. The term "substantially homologous to" as used in this invention encompasses such allelic variability as described in this paragraph.

The invention also includes a protein comprised of a predicted amino acid sequence for the mouse (SEQ ID NO:4) and human (SEQ ID NO:6) MSH<sup>R</sup> deduced from the nucleotide sequence comprising the complete coding sequence of the mouse (SEQ ID NO:3) and human (SEQ ID NO:5) MSH<sup>R</sup> gene as described herein.

In another aspect, the invention comprises a homogeneous composition of a 35.3 kilodalton mouse MSH<sup>R</sup> or derivative thereof, wherein the amino acid sequence of the MSH<sup>R</sup> or derivative thereof comprises the mouse MSH-R sequence shown in Figure 2 (SEQ ID NO:4).

In another aspect, the invention comprises a homogeneous composition of a 34.7 kilodalton human MSH<sup>R</sup> or derivative thereof, wherein the amino acid sequence of the MSH<sup>R</sup> or derivative thereof comprises the human MSH-R sequence shown in Figure 2 (SEQ ID NO:6).

This invention provides both nucleotide and amino acid probes derived from these sequences. The invention includes probes isolated from either cDNA or genomic DNA clones, as well as probes made synthetically with the sequence information derived therefrom. The invention specifically includes but is not limited to oligonucleotide, nick-translated, random primed, or *in vitro* amplified probes made using cDNA or genomic clone embodying the invention, and oligonucleotide and other synthetic probes synthesized chemically using the nucleotide sequence information of cDNA or genomic clone embodiments of the invention.

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It is a further object of this invention to provide sequences of mammalian MSH<sup>R</sup>, preferably the mouse or human MSH<sup>R</sup>, for use as nucleic acid hybridization probes to determine the pattern, amount and extent of expression of this receptor in various tissues of mammals, including humans. It is also an object of the present invention to provide nucleic acid hybridization probes derived from the sequences of the mouse or human MSH<sup>R</sup> to be used for the detection and diagnosis of genetic diseases. It is an object of this invention to provide nucleic acid hybridization probes derived from the DNA sequences of the mouse or human MSH<sup>R</sup> to be used for the detection of novel related receptor genes.

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The present invention also includes synthetic peptides made using the nucleotide sequence information comprising cDNA or genomic clone embodiments of the invention. The invention includes either naturally occurring or synthetic peptides which may be used as antigens for the production of MSH<sup>R</sup>-specific antibodies, or used for competitors of the MSH<sup>R</sup> molecule for drug binding, or to be used for the production of inhibitors of the binding of agonists or antagonists or analogues thereof to MSH<sup>R</sup> molecule.

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The present invention also provides antibodies against and epitopes of mammalian MSH<sup>R</sup>s, preferably mouse or human MSH<sup>R</sup> proteins. It is an object of the present invention to provide antibodies that is immunologically reactive to a mammalian MSH<sup>R</sup> protein. It is a particular object of the invention to provide a monoclonal antibodies to mammalian MSH<sup>R</sup> protein, most preferably mouse or

human MSH<sup>R</sup> protein.

It is also an object of the present invention to provide a hybridoma cell line that produces such an antibody. It is a particular object of the invention to provide a hybridoma cell line that is the result of fusion between a non-immunoglobulin producing mouse myeloma cell line and spleen cells derived from a mouse immunized with a human cell line which expresses MSH<sup>R</sup> antigen. The present invention also provides a hybridoma cell line that produces such an antibody, and that can be injected into a living mouse to provide an ascites fluid from the mouse that is comprised of such an antibody.

The present invention also provides a pharmaceutical composition comprising a therapeutically effective amount of a monoclonal antibody that is immunologically reactive to a mammalian MSH<sup>R</sup>, preferably a mouse or human MSH<sup>R</sup>, and in a pharmaceutically acceptable carrier.

It is a further object of the present invention to provide an epitope of a mammalian MSH<sup>R</sup> protein wherein the epitope is immunologically reactive to an antibody specific for the mammalian MSH<sup>R</sup>. In preferred embodiments, the epitope is derived from mouse of human MSH<sup>R</sup> protein.

It is another object of the invention to provide a chimeric antibody that is immunologically reactive to a mammalian MSH<sup>R</sup> protein. In a preferred embodiment, the chimeric antibody is a monoclonal antibody. In a preferred embodiment, the MSH<sup>R</sup> is a mouse or human MSH<sup>R</sup>.

The present invention provides a recombinant expression construct comprising the nucleotide sequence of a mammalian MSH<sup>R</sup>, preferably the mouse or human MSH<sup>R</sup> and sequences sufficient to direct the synthesis of mouse or human MSH<sup>R</sup> in cultures of transformed eukaryotic cells. In a preferred embodiment, the recombinant expression construct is comprised of plasmid sequences derived from the plasmid pcDNAI/neo and cDNA or genomic DNA of mouse or human MSH<sup>R</sup> gene. This invention includes a recombinant expression construct comprising essentially the nucleotide sequences of genomic or cDNA clones of mouse or human MSH<sup>R</sup> in an embodiment that provides for their expression in cultures of transformed eukaryotic cells.

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It is also an object of this invention to provide cultures of transformed eukaryotic cells that have been transformed with such a recombinant expression construct and that synthesize mammalian, preferably mouse or human, MSH<sup>R</sup> protein. In a preferred embodiment, the invention provides human 293 cells that synthesize mouse MSH<sup>R</sup>. In an additional preferred embodiment, the invention provides human 293 cells that synthesize human MSH<sup>R</sup> protein.

The present invention also includes protein preparations of mammalian. preferably mouse or human MSHR, and preparations of membranes containing mammalian MSH<sup>R</sup>, derived from cultures of transformed eukaryotic cells. In a preferred embodiment, cell membranes containing mouse MSH<sup>R</sup> protein are isolated from 293 cell cultures transformed with a recombinant expression construct that directs the synthesis of mouse MSH<sup>R</sup>. In another preferred embodiment, cell membranes containing human MSHR protein are isolated from 293 cell cultures transformed with a recombinant expression construct that directs It also an object of this invention to provide the synthesis of human MSH<sup>R</sup>. mammalian, preferably mouse or human MSH<sup>R</sup> for use in the in vitro screening of novel adenosine agonist and antagonist compounds. In a preferred embodiment, membrane preparations containing the mouse MSH<sup>R</sup>, derived from cultures of transformed eukaryotic cells, are used to determine the drug dissociation properties of various novel adenosine agonist and antagonist compounds in vitro. In another preferred embodiment, membrane preparations containing the human MSHR, derived from cultures of transformed eukaryotic cells, are used to determine the drug dissociation properties of various novel adenosine agonist and antagonist compounds in vitro. These properties are then used to characterize such novel compounds by comparison to the binding properties of known mouse or human MSH<sup>R</sup> agonists and antagonists.

The present invention will also be useful for the *in vivo* detection of analogues of agonists or antagonists of MSH<sup>R</sup>, known or unknown, either naturally occurring or as the embodiments of a drug.

It is an object of the present invention to provide a method for the quantitative detection of agonists or antagonists, or analogues thereof, of MSH<sup>R</sup>,

known or unknown, either naturally occurring or as the embodiments of a drug. It is an additional object of the invention to provide a method to detect such agonists, antagonists, or analogues thereof in blood, saliva, semen, cerebrospinal fluid, plasma, lymph, or any other bodily fluid.

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Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "melanocyte stimulating hormone receptor" as used herein refers to proteins substantially homologous to, and having substantially the same biological activity as, the protein coded for by the nucleotide sequence depicted in Figure 1 (SEQ ID NO:3). This definition is intended to encompass natural allelic variations in the melanocyte stimulating hormone receptor sequence. Cloned genes of the present invention may code for MSH<sup>R</sup>s of any species of origin, including, for example, mouse, rat, rabbit, cat, and human, but preferably code for receptors of mammalian, most preferably mouse and human, origin.

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Nucleic acid hybridization probes provided by the invention comprise DNA sequences that are substantially homologous to the DNA sequences in Figure 1A (SEQ ID NO:3) and 1B (SEQ ID NO:5). Nucleic acid probes are useful for detecting MSH<sup>R</sup> gene expression in cells and tissues using techniques well-known in the art, including but not limited to Northern blot hybridization, in situ hybridization and Southern hybridization to reverse transcriptase - polymerase chain reaction product DNAs. The probes provided by the present invention, including oligonucleotides probes derived therefrom, are useful are also useful for Southern hybridization of mammalian, preferably human, genomic DNA for screening for restriction fragment length polymorphism (RFLP) associated with certain genetic disorders.

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The production of proteins such as the MSH<sup>R</sup> from cloned genes by genetic engineering is well known. See, e.g., U.S. Patent No. 4,761,371 to Bell et al. at Col. 6 line 3 to Col. 9 line 65. (The disclosure of all U.S. patent references cited herein is to be incorporated herein by reference.) The discussion which follows is accordingly intended as an overview of this field, and is not intended to reflect the full state of the art.

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DNA which encodes the MSH<sup>R</sup> may be obtained, in view of the instant disclosure, by chemical synthesis, by screening reverse transcripts of mRNA from appropriate cells or cell line cultures, by screening genomic libraries from appropriate cells, or by combinations of these procedures, as illustrated below. Screening of mRNA or genomic DNA may be carried out with oligonucleotide

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probes generated from the MSH<sup>R</sup> gene sequence information provided herein. Probes may be labeled with a detectable group such as a fluorescent group, a radioactive atom or a chemiluminescent group in accordance with know procedures and used in conventional hybridization assays, as described in greater detail in the Examples below. In the alternative, MSH<sup>R</sup> gene sequences may be obtained by use of the polymerase chain reaction (PCR) procedure, with the PCR oligonucleotide primers being produced from the MSH<sup>R</sup> gene sequence provided herein. See U.S. Patent Nos. 4,683,195 to Mullis et al. and 4,683,202 to Mullis.

The MSH<sup>R</sup> may be synthesized in host cells transformed with a recombinant expression construct comprising a DNA sequence encoding the MSH<sup>R</sup>. Such a recombinant expression construct can also be comprised of a vector that is a replicable DNA construct. Vectors are used herein either to amplify DNA encoding the MSHR and/or to express DNA which encodes the MSH<sup>R</sup>. For the purposes of this invention, a recombinant expression construct is a replicable DNA construct in which a DNA sequence encoding the MSHR is operably linked to suitable control sequences capable of effecting the expression of the MSHR in a suitable host. The need for such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants.

Vectors useful for practicing the present invention include plasmids, viruses (including phage), retroviruses, and integratable DNA fragments (i.e., fragments integratable into the host genome by homologous recombination). The vector replicates and functions independently of the host genome, or may, in some instances, integrate into the genome itself. Suitable vectors will contain replicon and control sequences which are derived from species compatible with the

intended expression host. A preferred vector is the plasmid pcDNAI/neo. Transformed host cells are cells which have been transformed or transfected with recombinant expression constructs made using recombinant DNA techniques and comprising a mammalian MSH<sup>R</sup>. Transformed host cells may ordinarily express the mammalian MSH<sup>R</sup>, but host cells transformed for purposes of cloning or amplifying nucleic acid hybridization probe DNA need not express the receptor. When expressed, the mammalian MSH<sup>R</sup> will typically be located in the host cell membrane.

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DNA regions are operably linked when they are functionally related to each other. For example: a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of leaders sequences, contiguous and in the same translational reading frame.

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Cultures of cells derived from multicellular organisms are a desirable host for recombinant MSH<sup>R</sup> synthesis. In principal, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. However, mammalian cells are preferred, as illustrated in the Examples. Propagation of such cells in cell culture has become a routine procedure. See Tissue Culture, Academic Press, Kruse & Patterson, editors (1973). Examples of useful host cell lines are human 293 cells, VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and WI138, BHK, COS-7, CV, and MDCK cell lines. Human 293 cells are preferred. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located upstream from the gene to be expressed, along with a ribosome binding site, RNA splice sites (if introncontaining genomic DNA is used), a polyadenylation site, and a transcriptional termination sequence.

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An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral source (e.g., polyoma, adenovirus, VSV, or MPV), or may be provided by the host cell chromosomal replication mechanism. If the vector is

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integrated into the host cell chromosome, the latter may be sufficient.

The invention provides homogeneous compositions of mammalian MSH<sup>R</sup> protein produced by transformed eukaryotic cells as provided herein. Such homogeneous compositions are intended to be comprised of mammalian MSH<sup>R</sup> protein that comprises 90% of the protein in such homogeneous composition.

Mammalian MSH<sup>R</sup> protein made from cloned genes in accordance with the present invention may be used for screening agonist compounds for MSH<sup>R</sup> activity, or for determining the amount of a MSH<sup>R</sup> agonist or antagonist drug in a solution (e.g., blood plasma or serum). For example, host cells may be transformed with a recombinant expression construct of the present invention, MSH<sup>R</sup> expressed in that host, the cells lysed, and the membranes from those cells used to screen compounds for MSH<sup>R</sup> binding activity. Competitive binding assays in which such procedures may be carried out are well known in the art. By selection of host cells which do not ordinarily express MSH<sup>R</sup>s, pure preparations of membranes containing MSH<sup>R</sup>s can be obtained. Further, MSH<sup>R</sup> agonists and antagonists can be identified by transforming host cells with vectors of the present invention. Membranes obtained from such cells can be used in binding studies wherein the drug dissociation activity is monitored.

The recombinant expression constructs of the present invention are useful in molecular biology to transform cells which do not ordinarily express the MSH<sup>R</sup> to thereafter express this receptor. Such cells are useful as intermediates for making cell membrane preparations useful for receptor binding assays, which are in turn useful for drug screening. Further, genes and vectors comprising the recombinant expression construct of the present invention are useful in gene therapy. For such purposes, retroviral vectors as described in U.S. Patent No. 4,650,764 to Temin & Watanabe or U.S. Patent No. 4,861,719 to Miller may be employed. Cloned genes of the present invention, or fragments thereof, may also be used in gene therapy carried out homologous recombination or site-directed mutagenesis. See generally Thomas & Capecchi, 1987, Cell 51: 503-512; Bertling, 1987, Bioscience Reports 7: 107-112; Smithies et al., 1985, Nature 317: 230-234.

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Oligonucleotides of the present invention are useful as diagnostic tools for probing MSH receptor gene expression in tissues. For example, tissues can be probed *in situ* with oligonucleotide probes carrying detectable groups by conventional autoradiography techniques, as explained in greater detail in the Examples below, to investigate native expression of this receptor or pathological conditions relating thereto. Further, chromosomes can be probed to investigate the presence or absence of the MSH<sup>R</sup> gene, and potential pathological conditions related thereto, as also illustrated by the Examples below.

The invention also provides antibodies that are immunologically reactive to a mammalian MSH<sup>R</sup>. The antibodies provided by the invention can be raised in animals by inoculation with cells that express a mammalian MSH<sup>R</sup> or epitopes of a mammalian MSH<sup>R</sup> using methods well known in the art. Animals that can be used for such inoculations include individuals from species comprising cows, sheep, pigs, mice, rats, rabbits, hamsters, goats and primates. Preferred animals for inoculation are rodents (including mice, rats, hamsters) and rabbits. The most preferred animal is the mouse.

Cells that can be used for such inoculations, or for any of the other means used in the invention, include any cell line which naturally expresses a mammalian MSH<sup>R</sup>, or any cell or cell line that expresses a mammalian MSH<sup>R</sup> or any epitope therein as a result of molecular or genetic engineering, or that has been treated to increase the expression of a mammalian MSH<sup>R</sup> by physical, biochemical or genetic means. Preferred cells are human cells, most preferably human 293 cells that have been transformed with a recombinant expression construct comprising DNA sequences encoding a mammalian MSH<sup>R</sup> and that express the mammalian MSH<sup>R</sup> gene product.

The present invention provides monoclonal antibodies that are immunologically reactive with an epitope that is a mammalian MSH<sup>R</sup> present on the surface of mammalian cells, preferably human or mouse cells. These antibodies are made using methods and techniques well known to those of skill in the art.

Monoclonal antibodies provided by the present invention are produced by

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hybridoma cell lines, that are also provided by the invention and that are made by methods well known in the art. Hybridoma cell lines are made by fusing individual cells of a myeloma cell line with spleen cells derived from animals immunized with cells expressing a mammalian MSHR, including human cells, as described above. The myeloma cell lines used in the invention include lines derived from myelomas of mice, rats, hamsters, primates and humans. Preferred myeloma cell lines are from mouse, and the most preferred mouse myeloma cell line is P3X63-Ag8.653. The animals from whom spleens are obtained after immunization are rats, mice and hamsters, preferably mice, most preferably Balb/c mice. Spleen cells and myeloma cells are fused using a number of methods well known in the art, including but not limited to incubation with inactivated Sendai virus and incubation in the presence of polyethylene glycol (PEG). The most preferred method for cell fusion is incubation in the presence of a solution of 45% (w/v) PEG-1450. Monoclonal antibodies produced by hybridoma cell lines can be harvested from cell culture supernatant fluids from in vitro cell growth; alternatively, hybridoma cells can be injected subcutaneously and/or into the peritoneal cavity of an animal, most preferably a mouse, and the monoclonal antibodies obtained from blood and/or ascites fluid.

Monoclonal antibodies provided by the present invention can also be produced by recombinant genetic methods well known to those of skill in the art, and the present invention encompasses antibodies made by such methods that are immunologically reactive with an epitope of a mammalian MSH<sup>R</sup>.

The present invention encompasses fragments of the antibody that are immunologically reactive with an epitope of a mammalian MSH<sup>R</sup>. Such fragments can be produced by any number of methods, including but not limited to proteolytic cleavage, chemical synthesis or preparation of such fragments by means of genetic engineering technology. The present invention also encompasses single-chain antibodies that are immunologically reactive with an epitope of a mammalian MSH<sup>R</sup> made by methods known to those of skill in the art.

The present invention also encompasses an epitope of a mammalian MSH<sup>R</sup> that is comprised of sequences and/or a conformation of sequences present in the

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mammalian MSH<sup>R</sup> molecule. This epitope may be naturally occurring, or may be the result of proteolytic cleavage of the mammalian MSH<sup>R</sup> molecule and isolation of an epitope-containing peptide or may be obtained by synthesis of an epitope-containing peptide using methods well known to those skilled in the art. The present invention also encompasses epitope peptides produced as a result of genetic engineering technology and synthesized by genetically engineered prokaryotic or eukaryotic cells.

The invention also includes chimeric antibodies, comprised of immunologically reactive light chain and heavy chain peptides to an epitope that is a mammalian MSH<sup>R</sup>. The chimeric antibodies embodied in the present invention include those that are derived from naturally occurring antibodies as well as chimeric antibodies made by means of genetic engineering technology well known to those of skill in the art.

The Examples which follow are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

#### **EXAMPLE 1**

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# Isolation of an aMSH Receptor Probe by Random PCR Amplification of Human Melanoma cDNA Using Degenerate Oligonucleotide Primers

In order to clone novel G-protein coupled receptors, human melanoma cDNA was used as template for a polymerase chain reaction (PCR)-based random cloning experiment. PCR was performed using a pair of degenerate oligonucleotide primers corresponding to the putative third and sixth transmembrane regions of G-protein coupled receptors (Libert et al., 1989, Science 244: 569-72; Zhou et al., 1990, Nature 347: 76-80). The PCR products obtained in this experiment were characterized by nucleotide sequencing. Two novel sequences representing novel G-protein-coupled receptors were identified.

PCR amplification was performed as follows. Total RNA was isolated from a human melanoma tumor sample by the guanidinium thiocyanate method

(Chirgwin et al., 1979, Biochemistry 18: 5294-5299). Double-stranded cDNA was synthesized from total RNA with murine reverse transcriptase (BRL, Gaithersburg, MD) by oligo-dT priming [Maniatis et al., Molecular Cloning: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY), 1990]. The melanoma cDNA mixture was then subjected to 45 cycles of PCR amplification using 500 picomoles of degenerate oligonucleotide primers having the following sequence:

Primer III (sense):

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GAGTCGACCTGTG(C/T)G(C/T)(C/G)AT(C/T)(A/G)CIT(G/T)GAC(C/A)G(C/G)T AC

(SEQ ID NO:1)

15 and

Primer VI (antisense):

CAGAATTCAG(T/A)AGGGCAICCAGCAGAI(G/C)(G/A)(T/C)GAA (SEQ ID NO:2)

in 100  $\mu$ l of a solution containing 50 mM Tris-HCl (pH 8.3), 2.5 mM MgCl<sub>2</sub>, 0.01% gelatin, 200  $\mu$ M each dNTP, and 2.5 units of Taq polymerase (Saiki et al., 1988, Science 239: 487-491). These primers were commercially synthesized by Research Genetics Inc. (Huntsville, AL). Each PCR amplification cycle consisted of incubations at 94°C for 1 min (denaturation), 45°C for 2 min (annealing), and 72°C for 2 min (extension).

Amplified products of the PCR reaction were extracted with phenol/chloroform and precipitated with ethanol. After digestion with *EcoRI* and *SaII*, the PCR products were separated on a 1.2% agarose gel. A slice of this gel, corresponding to PCR products of 300 basepairs (bp) in size, was cut out and purified using glass beads and sodium iodide, and the insert was then cloned into a pBKS cloning vector (Stratagene, LaJolla, CA).

A total of 172 of such pBKS clones containing inserts were sequenced using Sequenase (U. S. Biochemical Corp., Cleveland, OH) by the dideoxynucleotide chain termination method (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA 74: 5463-5467). Two types of sequences homologous to other

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G-protein coupled receptors were identified.

#### **EXAMPLE 2**

#### Isolation and Sequence Analysis of Mouse aMSH Receptor cDNA

Probes isolated in Example 1 was used to screen a Cloudman melanoma cDNA library in order to isolate a full-length cDNA corresponding to the cloned probe. One clone was isolated from a library of 5 x 10° clones screened as described below. This clone contained an insert of 2.6 kilobases (kb). The nucleotide sequence of the complete coding region was determined, as shown in Figure 1A (SEQ ID NO:3).

The PCR probe was labeled by the random-priming method (Stratagene PrimeIt, #300387, LaJolla, CA) and used to screen a Cloudman melanoma line cDNA library constructed in the λZAP vector (Stratagene). Library screening was performed using techniques well-known in the art as described in Bunzow et al. (1988, Nature 336: 783-787) at moderate stringency (40% formamide, 1M NaCl, 50mM Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, 0.2% sodium dodecyl sulfate, 100μg/ml salmon sperm DNA, 10X Denhardt's solution). One cDNA clone was identified (termed mmelA) and its 2.6 kb cDNA insert was isolated and subcloned into pBKS (Stratagene); the resulting plasmid was called pmmelA. Nucleotide sequence analysis and homology comparisons were done on the OHSU computer system with software provided by Intelligenetics Inc. (Mountain View, CA).

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The nucleotide sequence of pmmelA (the cDNA clone isolated as described above) is shown in Figure 1A (SEQ ID NO:3). The longest open reading frame of this cDNA encodes a predicted protein product of 315 amino acids with a calculated molecular weight of 35.3 kilodaltons (kD). The deduced amino acid sequence is shown in Figure 2 (SEQ ID NO:4) as mouse MSH-R. Single letter amino acid codes are used [see, G. Zubay, Biochemistry (2d ed.), 1988 (MacMillen Publishing: New York) p.33]. Uppercase lettering indicates amino acid residues in common between the receptor proteins shown; lowercase lettering indicates divergent residues.

Hydrophobicity analysis (Kyte & Doolittle, 1982, J. Mol. Biol. <u>157</u>: 105-132) of the deduced amino acid sequence showed that the protein contains seven hydrophobic stretches of 21 to 26 amino acids apiece. Putative transmembrane domains are overlined and designated with Roman numerals.

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#### **EXAMPLE 3**

# Construction of Mouse αMSH<sup>R</sup> Expression Plasmids, DNA Transfection and Functional Expression of the αMSH<sup>R</sup> Gene Product

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In order to biochemically characterize the putative mouse  $\alpha MSH^R$  cDNA isolated as in Example 2, and to confirm that it encodes an  $\alpha MSH$  receptor, mmelA was cloned into a mammalian expression vector, this vector transfected into human 293 cells, and cell lines generated that expressed the putative  $\alpha MSH^R$  receptor at the cell surface. Such cells and membranes isolated from such cells were used for biochemical characterization experiments described below.

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The entire coding region of the  $\alpha$ MSH<sup>R</sup> cDNA insert from mmelA contained in a 2.1kb fragment was excised from pBSK and subcloned into the *BamHI/XhoI* sites of pcDNAI/neo expression vector (Invitrogen, San Diego, CA). The resulting plasmid was called pcDNA-mmelA. pcDNA-mmelA plasmid DNA was prepared in large-scale through one cycle of CsCl gradient ultracentrifugation and 20  $\mu$ g pcDNA-mmelA DNA were transfected into each 100mm dish of 293 cells using the calcium phosphate method (*see* Chen & Okayama, 1987, Mol. Cell. Biol. 7: 2745-2752). After transfection, cells were cultured in DMEM media supplemented with 10% calf serum in a 3% CO<sub>2</sub> atmosphere at 37°C. Selection was performed with neomycin (G418; GIBCO) at a concentration of 1000  $\mu$ g/ml; selection was started 72 hr after transfection and continued for 3 weeks.

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The αMSH<sup>R</sup> is known to couple to G-proteins and thereby activate adenyl cyclase, increasing intracellular levels of cAMP (see Buckley & Ramachandran, 1981, Proc. Natl. Acad. Sci. USA 78: 7431-7435; Grahame-Smith et al., 1967, J. Biol. Chem 242: 5535-5541; Mertz & Catt, 1991, Proc. Natl. Acad. Sci. USA 88: 8525-8529; Pawalek et al., 1976, Invest. Dermatol. 66: 200-209). This

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property of cells expressing the  $\alpha$ MSH receptor was used analyze expression of the  $\alpha$ MSH receptor in cell colonies transfected with the expression vectors described herein as follows. Cells ( $\sim 1 \times 10^6$ ) were plated in 6-well dishes, washed once with DMEM containing 1% bovine serum albumin (BSA) and 0.5mM IBMX (a phosphodiesterase inhibitor), then incubated for 45 minutes at 37°C with varying concentrations of the melanotropic peptides  $\alpha$ MSH,  $\beta$ MSH,  $\gamma$ MSH, the MSH peptide analogues Nle<sup>4</sup>, D-Phe<sup>7</sup>- $\alpha$ MSH (NDP-MSH), and ACTH. Following hormone treatment, the cells were washed twice with phosphate buffered saline and intracellular cAMP extracted by lysing the cells with 1ml of 60% ethanol. Intracellular cAMP concentrations were determined using an assay (Amersham) which measures the ability of cAMP to displace [8-³H] cAMP from a high affinity cAMP binding protein (see Gilman, 1970, Proc. Natl. Acad. Sci. USA 67: 305-312).

The results of these experiments are shown in Figure 3. The abscissa indicates the concentration of each hormone and the ordinate indicates the percentage of basal intracellular cAMP concentration achieved by each treatment. Points indicate the mean of duplicate incubations; the standard error did not exceed 15% for any data point. None of the peptides tested induced any change in intracellular cAMP in cells containing the vector alone. Cells expressing the murine aMSH receptor responded to melanotropic peptides with a 2-3 fold elevation of intracellular cAMP, similar to levels of cAMP induced by these peptides in the Cloudman cell line (see Pawalek, 1985, Yale J. Biol. Med. 58; 571-578). The EC<sub>50</sub> values determined for  $\alpha$ MSH (2.0x10-9M), ACTH (8.0x10<sup>-9</sup>M) and the superpotent MSH analogue NDP-MSH (2.8x10<sup>-11</sup>M) correspond closely to reported values (see Tatro et al., 1990, Cancer Res. 50: 1237-1242). As expected, the  $\beta$ MSH peptide had an EC<sub>50</sub> value comparable to  $\alpha$ MSH<sup>22</sup> while  $\gamma$ MSH had little or no activity (see Slominski et al., 1992, Life Sci. 50: 1103-1108), confirming the identity of this receptor as a melanocyte aMSH receptor.

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#### **EXAMPLE 4**

#### Isolation and Characterization of a Human aMSHR Genomic Clone

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In order to isolate a human counterpart of the murine melanocyte  $\alpha$ MSH receptor gene, a human genomic library was screened at high stringency (50% formamide, 42°C) using the human PCR fragments isolated as described in Example 1. Two different types of sequences were isolated, corresponding to the two PCR fragments, and were found to encode highly related G protein-coupled receptors. These genomic clones were sequenced as described in Example 2. One of these genomic clones was determined to encode an human MSH receptor (SEQ ID NO:5). The human MSH receptor has a predicted amino acid sequence (SEQ ID NO:6) that is 75% identical and colinear with the mouse  $\alpha$ MSH receptor cDNA sequence (Figure 2), represented as human MSH-R. The predicted molecular weight of the human MSH<sup>R</sup> is 34.7kD.

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The predicted amino acid sequences of the mouse  $\alpha$ MSH<sup>R</sup> (SEQ ID NO:4) and human MSHR (SEQ ID NO:6) are aligned in Figure 2. These sequences define the melanocortin receptors as a novel subfamily of the G protein-coupled receptors with a number of unusual features. The melanocortin receptors are the smallest G protein-coupled receptors identified to date (297-317aa) resulting from a short amino terminal extracellular domain, a short carboxy-terminal intracellular domain, and a very small third intracellular loop. The melanocortin receptors are lack several amino acid residues present in most G protein coupled receptors (see Probst et al., 1992, DNA & Cell Biol. 11: 1-20), including the proline residues in the 4th and 5th transmembrane domains, likely to introduce a bend in the alpha helical structure of the transmembrane domains and thought to be involved in the formation of the binding pocket (see Applebury & Hargrave, 1986, Vision Res. 26: 1881-1895), and one or both of the cysteine residues thought to form a disulfide bond between the first and second extracellular loops (see Dixon et al., 1987, EMBO J. 6: 3269-3275 and Karnik et al., 1988, Proc. Natl. Acad. Sci. USA 85: 8459-8463). Remarkably, the melanocortin receptors do not appear highly related to the other G protein-coupled receptors which recognize peptide ligands, such as the receptors for bombesin (see Spindel et al., 1990, Mol.

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Endocrinol. 4: 1956-1963) or substance K (see Masu et al., 1987, Nature 329: 836-838) but rather, are more closely related to the receptor for  $\Delta^9$ -tetradhydrocannabinol (see Matsuda et al., 1990, Nature 346: 561-564). The cannabinoid receptor also lacks the conserved proline in transmembrane 5 and the cysteine in the first extracellular loop necessary for disulfide bond formation. Least parsimony analysis with the receptor sequences shown in Figure 2 suggests the cannabinoid and melanocortin receptors may be evolutionarily related and form a subfamily distinct from the peptide receptors and the amine receptors. Regardless of whether the similarities are the result of evolutionary conservation or convergence, the sequence and putative structural similarities between the melanocortin and cannabinoid receptors may be informative in the search for the endogenous cannabinoid-like ligand.

#### **EXAMPLE 5**

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#### Tissue Distribution of aMSH Receptors

To further gain insight into these receptors, we have examined the tissue distribution of their corresponding mRNAs from various tissues by performing Northern hybridization experiments on RNA isolated from various tissues (see Maniatis et al., ibid.). The results of these experiments are shown in Figure 4.

A panel of tissue samples was examined by Northern hybridization analysis performed under high stringency conditions. The same nitrocellulose filter was hybridized successively with a human MSH receptor probe and a mouse MSH receptor probe to determine the distribution of each receptor mRNA. The murine MSH receptor is encoded predominantly by a single mRNA species of 3.9kb, while the human MSH receptor is encoded, in two melanoma samples, predominantly by a 3.0kb species. High levels of receptor mRNA are seen in both primary mouse melanocytes and mouse melanoma cell lines. In contrast, extremely low levels of receptor mRNA were detected in primary human melanocytes, and many human melanoma samples (see melanoma 1, Fig. 4). Most intriguing is the dramatic elevation of MSH-R mRNA seen thus far in 3 of 11 samples tested, such as is seen in melanoma sample #2 (Fig. 4).

Additionally, we have been unable to detect expression in the brain of any of the receptors described here, despite extensive documentation of MSH binding sites there as well as in other tissues. These finding suggest the existence of alternate forms of these or related receptors that may be specifically expressed in brain tissue.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

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-24-

#### SEQUENCE LISTING

- (1) GENERAL INFORMATION:
  - (i) APPLICANT: Cone, Roger D
    Mountjoy, Kathleen G
  - (ii) TITLE OF INVENTION: Melanocyte Stimulating Hormone Receptor and Uses
  - (iii) NUMBER OF SEQUENCES: 6
  - (iv) CORRESPONDENCE ADDRESS:
    - (A) ADDRESSEE: Allegretti & Witcoff, Ltd.
    - (B) STREET: 10 South Wacker Drive, Suite 3000
    - (C) CITY: Chicago
    - (D) STATE: Illinois
    - (E) COUNTRY: USA
    - (F) ZIP: 60606
  - (v) COMPUTER READABLE FORM:
    - (A) MEDIUM TYPE: Floppy disk
    - (B) COMPUTER: IBM PC compatible
    - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
    - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
  - (vi) CURRENT APPLICATION DATA:
    - (A) APPLICATION NUMBER: PCT/US93/03247
    - (B) FILING DATE: 07-APR-1993
    - (C) CLASSIFICATION:
  - (viii) ATTORNEY/AGENT INFORMATION:
    - (A) NAME: Noonan, Kevin E
    - (B) REGISTRATION NUMBER: 35,303
    - (C) REFERENCE/DOCKET NUMBER: 92,154-A
    - (ix) TELECOMMUNICATION INFORMATION:
      - (A) TELEPHONE: 312-715-1000
      - (B) TELEFAX: 312-715-1234
      - (C) TELEX: 910-221-5317
- (2) INFORMATION FOR SEQ ID NO:1:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 33 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: DNA (genomic)
  - (ix) FEATURE:
    - (A) NAME/KEY: misc feature

-25-

			LOCATION: OTHER INFO				e	
	(xi)	SEQU	ENCE DESCR	IPTION: SI	EQ ID N	0:1:		
GAG	TCGAC	CT GI	GYGYSATY RO	CTKGACMGS	TAC			33
(2)	INFO	RMATI	ON FOR SEQ	ID NO:2:				
	(i)	(A) (B) (C)	ENCE CHARAC LENGTH: 31 TYPE: nucl STRANDEDNE TOPOLOGY:	base pai leic acid ESS: sing	irs			
	(ii)	MOLE	CULE TYPE:	DNA (geno	mic)			
	(ix)	·(B)	NAME/KEY: LOCATION: OTHER INFO	131	/functi		e	
	(xi)	SEQU	ENCE DESCRI	PTION: SE	EQ ID NO	D:2:		
CAG	AATTC	ag wa	GGGCACCA GC	CAGASRYGA	A			31
(2)	INFO	RMATI	ON FOR SEQ	ID NO:3:				
	(i)	(A) (B) (C)	ENCE CHARAC LENGTH: 12 TYPE: nucl STRANDEDNE TOPOLOGY:	60 base p leic acid ESS: singl	pairs			
	(ii)	MOLE	CULE TYPE:	CDNA				
	(ix)		URE: NAME/KEY: LOCATION:					
	(ix)	FEAT	URE: NAME/KEY:	5/11 <b>T</b> P				
		(A) (B)	LOCATION:					

(ix) FEATURE:

(A) NAME/KEY: 3'UTR
(B) LOCATION: 960..1260

A	вn	Ala	Thr	Ser 20	His	Leu	Gly	Leu	Ala 25	Thr	Asn	Gln	Ser	Glu 30	Pro	Trp
C	yв	Leu	Tyr 35	Val	Ser	Ile	Pro	<b>Авр</b> 40	Gly	Leu	Phe	Leu	Ser 45	Leu	Gly	Leu
V.	al	Ser 50	Leu	Val	Glu	Asn	Val 55	Leu	Val	Val	Ile	Ala 60	Ile	Thr	Lув	Asn
	rg 65	Asn	Leu	His	Ser	Pro 70	Met	Tyr	Tyr	Phe	Ile 75	Cys	Сув	Leu	Ala	Leu 80
S	er	Asp	Leu	Met	Val 85	Ser	Val	Ser	Ile	<b>Val</b> 90	Leu	Glu	Thr	Thr	11e 95	Ile
L	eu	Leu	Leu	Glu 100	Val	Gly	Ile	Leu	<b>Val</b> 105	Ala	Arg	Val	Ala	Leu 110	Val	Gln
G	ln	Leu	Asp 115	Asn	Leu	Ile	Asp	Val 120	Leu	Ile	Сув	Gly	Ser 125	Met	Val	Ser
S	er	Leu 130	Сув	Phe	Leu	Gly	Ile 135	Ile	Ala	Ile	Asp	Arg 140	Tyr	Ile	Ser	Ile
P)		Tyr	Ala	Leu	Arg	Tyr 150	His	Ser	Ile	Val	Thr 155	Leu	Pro	Arg	Ala	<b>A</b> rg 160
A:	rg	Ala	Val	Val	Gly 165	Ile	Trp	Met	Val	Ser 170	Ile	Val	Ser	Ser	Thr 175	Leu
P	he	Ile	Thr	Tyr 180	Tyr	Lys	His	Thr	Ala 185	Val	Leu	Leu	Сув	Leu 190	Val	Thr
P	he	Phe	Leu 195	Ala	Met	Leu	Ala	Leu 200	Met	Ala	Ile	Leu	Tyr 205	Ala	His	Met
P	he	Thr 210	Arg	Ala	Сув	Gln	His 215	Val	Gln	Gly	Ile	Ala 220	Gln	Leu	His	Lys
	rg 25	Arg	Arg	Ser	Ile	<b>Arg</b> 230	Gln	Gly	Phe	Сув	Leu 235	Lys	Gly	Ala	Ala	Thr 240
L	eu	Thr	Ile	Leu	Leu 245	Gly	Ile	Phe	Phe	Leu 250	Сув	Trp	Gly	Pro	Phe 255	Phe
L	eu	His	Leu	Leu 260	Leu	Ile	Val	Leu	Сув 265	Pro	Gln	His	Pro	Thr 270	Сув	Ser
C	ys	Ile	Phe 275	Lys	Asn	Phe	Asn	Leu 280	Phe	Leu	Leu	Leu	Ile 285	Val	Leu	Ser
S	er	Thr	Val	Asp	Pro	Leu	Ile	Tyr	Ala	Phe	Arg	Ser	Gln	Glu	Leu	Arg

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Met	Thr	Leu	Lys	Glu	Val	Leu	Leu	Сув	Ser	Trp
305					310					315

### (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1633 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 462..1415
- (ix) FEATURE:
  - (A) NAME/KEY: 5'UTR
  - (B) LOCATION: 1..461
- (ix) FEATURE:
  - (A) NAME/KEY: 3'UTR
  - (B) LOCATION: 1416..1633

### (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCCGCATGTG GCCGCCCTCA ATGGAGGGCT CTGAGAACGA CTTTTAAAAC GCAGAGAAAA	60
AGCTCCATTC TTCCCAGACC TCAGCGCAGC CCTGGCCCAG GAAGGGAGGA GACAGAGGCC	120
AGGACGGTCC AGAGGTGTCG AAATGTCCTG GGAACCTGAG CAGCAGCCAC CAGGGAAGAG	180
GCAGGGAGGG AGCTGAGGAC CAGGCTTGGT TGTGAGAATC CCTGAGCCCA GGCGGTTGAT	240
GCCAGGAGGT GTCTGGACTG GCTGGGCCAT GCCTGGGCTG ACCTGTCCAG CCAGGGAGAG	300
GGTGTGAGGG CAGATCTGGG GGTGCCCAGA TGGAAGGAGG CAGGCATGGG GACACCCAAG	360
GCCCCCTGGC AGCACCATGA ACTAAGCAGG ACACCTGGAG GGGAAGAACT GTGGGGACCT	420
GGAGGCCTCC AACGACTCCT TCCTGCTTCC TGGACAGGAC T ATG GCT GTG CAG Met Ala Val Gln 1	473
GGA TCC CAG AGA AGA CTT CTG GGC TCC CTC AAC TCC ACC CCC ACA GCC Gly Ser Gln Arg Arg Leu Leu Gly Ser Leu Asn Ser Thr Pro Thr Ala	521

ATC CCC CAG CTG GGG CTG GCC AAC CAG ACA GGA GCC CGG TGC CTG

1le Pro Gln Leu Gly Leu Ala Ala Asn Gln Thr Gly Ala Arg Cys Leu
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GAG	GTG	TCC	ATC	TCT	GAC	GGG	CTC	TTC	CTC	AGC	CTG	GGG	CTG	GTG	AGC	617
					Asp											
_					CTG Leu											665
					TAC Tyr											713
					ACG Thr 90											761
					CTG Leu											809
					GTG Val											857
					ATC Ile											905
					AGC Ser											<b>95</b> 3
			_		GTG Val 170	_										1001
					GTG Val											1049
					CTC Leu											1097
		_			GCC Ala				_							1145
					GGC Gly											1193

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ATC Ile 245	CTG Leu	CTG Leu	GGC Gly	ATT Ile	TTC Phe 250	TTC Phe	CTC Leu	TGC Cys	TGG Trp	GGC Gly 255	CCC Pro	TTC Phe	TTC Phe	CTG Leu	CAT His 260	1241
CTC Leu	ACA Thr	CTC Leu	ATC	GTC Val 265	CTC Leu	TGC Cys	ccc Pro	GAG Glu	CAC His 270	CCC Pro	ACG Thr	TGC Сув	GGC Gly	TGC Cys 275	ATC Ile	1289
TTC Phe	AAG Lys	AAC Aan	TTC Phe 280	AAC Asn	CTC Leu	TTT Phe	CTC Leu	GCC Ala 285	CTC Leu	ATC Ile	ATC Ile	TGC Cys	AAT Asn 290	GCC Ala	ATC Ile	1337
ATC Ile	GAC Asp	ccc Pro 295	CTC Leu	ATC Ile	TAC Tyr	GCC Ala	TTC Phe 300	CAC His	AGC Ser	CAG Gln	GAG Glu	CTC Leu 305	CGC Arg	AGG Arg	ACG Thr	1385
Leu			GTG Val		Thr				TGAG	CGCG	GT G	CACG	:CGCT	T		1432
TAAG	TGTG	CT G	GGCA	GAGG	G AG	GTGG	TGAT	ATT	GTGG	TCT	GGTT	CCTG	TG I	GACC	CTGGG	1492
CAGT	TCCI	TA C	CTCC	CTGG	т сс	CCGT	TTGT	CAA	AGAG	GAT	GGAC	TAAA	TG A	TCTC	TGAAA	1552
GTGT	TGAA	cc c	CGGA	CCCT	т ст	GGGC	AGGG	AGG	GGTC	CTG	CAAA	ACTO	CA G	GCAG	GACTT	1612
CTCA	CCAG	CA G	TCGT	GGGA	A C											1633

- (2) INFORMATION FOR SEQ ID NO:6:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 317 amino acids
    - (B) TYPE: amino acid
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: protein
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Ala Val Gln Gly Ser Gln Arg Arg Leu Leu Gly Ser Leu Asn Ser 1 5 10 15

Thr Pro Thr Ala Ile Pro Gln Leu Gly Leu Ala Ala Asn Gln Thr Gly 20 25 30

Ala Arg Cys Leu Glu Val Ser Ile Ser Asp Gly Leu Phe Leu Ser Leu
35 40 45

Gly Leu Val Ser Leu Val Glu Asn Ala Leu Val Val Ala Thr Ile Ala 50 55 60

Lys Asn Arg Asn Leu His Ser Pro Met Tyr Cys Phe Ile Cys Cys Leu 65 70 75 80

Ala	Leu	Ser	Asp	Leu 85	Leu	Val	Ser	Gly	Thr 90	Asn	Val	Leu	Glu	Thr 95	Ala
Val	Ile	Leu	Leu 100	Leu	Glu	Ala	Gly	Ala 105	Leu	Val	Ala	Arg	Ala 110	Ala	Val
Leu	Gln	Gln 115	Leu	Авр	Asn	Val	11e 120	Авр	Val	Ile	Thr	Сув 125	Ser	ser	Met
Leu	Ser 130	Ser	Leu	Сув	Phe	Leu 135	Gly	Ala	Ile	Ala	Val 140	Авр	Arg	Tyr	Ile
Ser 145	Ile	Phe	Tyr	Ala	Leu 150	Arg	Tyr	His	Ser	11e 155	Val	Thr	Leu	Pro	<b>A</b> rg 160
Ala	Pro	Arg	Ala	Val 165	Ala	Ala	Ile	Trp	Val 170	Ala	Ser	Val	Val	Phe 175	Ser
Thr	Leu	Phe	11e 180	Ala	Tyr	Tyr	Авр	His 185	Val	Ala	Val	Leu	Leu 190	Сув	Leu
Val	Val	Phe 195	Phe	Leu	Ala	Met	Leu 200	Val	Leu	Met	Ala	Val 205	Leu	Tyr	Val
His	Met 210	Leu	Ala	Arg	Ala	Сув 215	Gln	His	Ala	Gln	Gly 220	Ile	Ala	Arg	Leu
His 225	Lys	Arg	Gln	Arg	Pro 230	Val	His	Gln	Gly	Phe 235	Gly	Leu	Lys	Gly	Ala 240
Val	Thr	Leu	Thr	11e 245	Leu	Leu	Gly	Ile	Phe 250	Phe	Leu	Сув	Trp	Gly 255	Pro
Phe	Phe	Leu	His 260	Leu	Thr	Leu	Ile	Val 265	Leu	Суз	Pro	Glu	His 270	Pro	Thr
Сув	Gly	Сув 275	Ile	Phe	Lys	Asn	Phe 280	Asn	Leu	Phe	Leu	Ala 285	Leu	Ile	Ile
Сув	Asn 290	Ala	Ile	Ile	Asp	Pro 295	Leu	Ile	Tyr	Ala	Phe 300	His	Ser	Gln	Glu
Leu 305	Arg	Arg	Thr	Leu	Lys 310	Glu	Val	Leu	Thr	Сув 315	Ser	Trp			

#### WHAT WE CLAIM IS:

- l. A nucleic acid comprising a nucleotide sequence encoding a mammalian melanocyte stimulating hormone receptor.
- 2. A nucleic acid according to Claim 1 wherein the mammalian melanocyte stimusating hormone receptor is the mouse melanocyte stimulating hormone receptor.
- 3. A nucleic acid according to Claim 1 wherein the nucleotide sequence is substantially homologous to the sequence in Figure 1A (SEQ ID NO:3).
- 4. A nucleic acid according to Claim 1 wherein the mammalian melanocyte stimulating hormone receptor is the human melanocyte stimulating hormone receptor.
- 105. A nucleic acid according to Claim I wherein the nucleotide sequence is substantially homologous to the sequence in Figure 1B (SEQ ID NO:5).
- 6. A DNA sequence according to Claim 1 wherein the mammalian melanocyte stimulating hormone receptor encoded therein has the melanotropic peptide response properties described in Figure 3.
- 157. A homogeneous composition of a 35.3 kilodalton melanocyte stimulating hormone receptor or derivative thereof, wherein the amino acid sequence of the melanocyte stimulating hormone receptor or derivative thereof comprises the mouse MSH-R sequence shown in Figure 2 (SEQ ID NO:4).
- 8. A homogeneous composition of a 34.6 kilodalton melanocyte stimulating horm200 receptor or derivative thereof, wherein the amino acid sequence of the melanocyte stimulating hormone receptor or derivative thereof comprises the human MSH-R sequence shown in Figure 2 (SEQ ID NO:6).
- 9. A nucleic acid hybridization probe for the detection of mammalian melanocyte stimulating hormone receptor expression comprising the nucleotide sequênce of Claim 3.
- 10. The nucleic acid hybridization probe according to Claim 9 whereby the probe is adapted for use in the detection and diagnosis of genetic disease in a human.
- 11. The nucleic acid hybridization probe according to Claim 9 whereby the proba0 is adapted for use in the detection, isolation and characterization of novel mammalian receptor genes.
- 12. A nucleic acid hybridization probe for the detection of mammalian melanocyte stimulating hormone receptor expression comprising the nucleotide sequence of Claim 5.
- 35 13. The nucleic acid hybridization probe according to Claim 12 whereby the probe is adapted for use in the detection and diagnosis of genetic disease in a

human.

- 14. The nucleic acid hybridization probe according to Claim 12 whereby the probe is adapted for use in the detection, isolation and characterization of novel mammalian receptor genes.
- 515. A recombinant expression construct comprising a nucleotide sequence encoding a mammalian melanocyte stimulating hormone receptor.
- 16. A recombinant expression construct comprising the DNA sequence of Claim 3, wherein the construct is capable of expressing the mouse melanocyte stimulating hormone receptor in a transformed eukaryotic cell culture.
- 1017. A recombinant expression construct comprising the DNA sequence of Claim 5, wherein the construct is capable of expressing the human melanocyte stimulating hormone receptor in a transformed eukaryotic cell culture.
- 18. The recombinant expression construct of Claim 15 comprising pcDNAI/neo sequences.
- 15 19. A eukaryotic cell culture transformed with the expression construct of Claim 16, wherein the transformed eukaryotic cell culture is capable of expressing mouse melanocyte stimulating hormone receptor.
- 20. A eukaryotic cell culture transformed with the expression construct of Claim 17, wherein the transformed eukaryotic cell culture is capable of expressing the Edman melanocyte stimulating hormone receptor.
- 21. A method of screening a compound as an inhibitor of agonist binding to a mammalian melanocyte stimulating hormone receptor, the method comprising the following steps:
  - (a) transforming a eukaryotic cell culture with an expression construct as
  - in Claim 15 capable of expressing the melanocyte stimulating hormone receptor in a eukaryotic cell; and
    - (b) assaying for ability of the compound to inhibit the binding of a detectable melanocyte stimulating hormone receptor agonist.
- 22. The method of Claim 21 wherein the mammalian melanocyte stimulating hormôde receptor is the mouse melanocyte stimulating hormone receptor.
- 23. The method of Claim 21 wherein the mammalian melanocyte stimulating hormone receptor is the human melanocyte stimulating hormone receptor.
- 24. A method of quantitatively detecting a compound as an inhibitor of agonist binding to a mammalian melanocyte stimulating hormone receptor, the method complising the following steps:
  - (a) transforming a eukaryotic cell culture with an expression construct as

- in Claim 15 capable of expressing the mammalian melanocyte stimulating hormone receptor in a eukaryotic cell; and
- (b) assaying for amount of a compound by measuring the extent of inhibition of binding of a detectable receptor agonist.
- 525. The method of Claim 24 wherein the mammalian melanocyte stimulating hormone receptor is the mouse melanocyte stimulating hormone receptor.
- 26. The method of Claim 24 wherein the mammalian melanocyte stimulating hormone receptor is the human melanocyte stimulating hormone receptor.
- 27. The method of Claim 24 wherein the compound to be tested is present in a human.
  - 28. The method of Claim 24 wherein the compound is present in human blood.
- 29. The method of Claim 24 wherein the compound is present in human cerebrospinal fluid.
  - 30. The method of Claim 24 wherein the compound is unknown.
- 1531. An antibody or fragment thereof that is immunologically reactive to a mammalian melanocyte stimulating hormone receptor.
- 32. The antibody according to Claim 31, wherein the antibody is a monoclonal antibody.
- 33. The antibody according to Claim 31, wherein the mammalian melanocyte stim20ating hormone receptor is the mouse melanocyte stimulating hormone receptor.
- 34. The antibody according to Claim 31, wherein the mammalian melanocyte stimulating hormone receptor is the human melanocyte stimulating hormone receptor.
- 35. A cell line which produces an antibody or fragment thereof that is immunologically reactive to a mammalian melanocyte stimulating hormone receptor.
- 2536. The cell line according to Claim 35, wherein the antibody is a monoclonal antibody.
- 37. The cell line according to Claim 35, wherein the mammalian melanocyte stimulating hormone receptor is the mouse melanocyte stimulating hormone receptor.
- 38. The cell line according to Claim 35, wherein the mammalian melanocyte stimbOating hormone receptor is the human melanocyte stimulating hormone receptor.
- 39. A pharmaceutical composition comprising a therapeutically effective amount of an antibody or fragment thereof according to claim 31 in a pharmaceutically acceptable carrier.
- 40. An epitope of a mammalian melanocyte stimulating hormone receptor wherain the epitope is immunologically reactive to the antibody or fragment thereof according to claim 31.

- 41. The epitope according to claim 40 wherein the mammalian melanocyte stimulating hormone receptor is the mouse melanocyte stimulating hormone receptor.
- 42. The epitope according to claim 40 wherein the mammalian melanocyte stimulating hormone receptor is the human melanocyte stimulating hormone receptor.
- 543. A chimeric antibody that is immunologically reactive to a mammalian melanocyte stimulating hormone receptor.
- 44. The chimeric antibody according to claim 43 wherein the mammalian melanocyte stimulating hormone receptor is the mouse melanocyte stimulating hormone receptor.
- 1045. The chimeric antibody according to claim 43 wherein the mammalian melanocyte stimulating hormone receptor is the human melanocyte stimulating hormone receptor.

# Figure 1A

	10	20	30	40	50	60	70
TICCIG	ACAA	GACTATGTC	C ACTCAGGAGG	CCCAGAAGAC	TCTTCTGGGT	TCTCTCAACT	CCAATGCCAC
(TCTCA(	80	90 GGACTGGCC	100		120	130	140 AGATGGCCTC
						IGICCATCCC	AGATGGCCTC
	.50 .GCC	160 TAGGGCTGG	170 F GAGTCTGGTG	180 GAGAATGTGC	190 TGGTTGTGAT	200 AGCCATCACC	210
	20		240	250			
			TACTICATET	GCTGCCTGGC	260 CCTGTCTGAC	270 CTGATGGTAA	280 GTGTCAGCAT
	90	300		320	330	340	350
CGTGCTG	GAG		TCCTGCTGCT		ATCCTGGTGG	CCAGAGTGGC	TTTGGTGCAG
	60	370	380	390	400	410	420
CAGCTGG	ACA	ACCTCATTGA	CGTGCTCATC	TGTGGCTCCA	TGGTGTCCAG	TCTCTGCTTC	CTGGGCATCA
	30	440	450	460	470	480	490
		CCGCTACATC	TCCATCTTCT				TGCCCAGAGC
	DO GCT	510 GTCGTGGGCA	520 TCTGGATGGT	530	540	550 TCTTTATCAC	560
						ICITIATUAC	CIACIACAAG
	70 CCG	580 TTCTGCTCTG	590 CCTCGTCACT	600 TTCTTTCTAG	610 CCATGCTGGC	620 ACTCATGGCG	630 ATTCTCTATC
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		CACGAGAGCG	TGCCAGCACG	TCCAGGGCAT	TGCCCAGCTC	CACAAAAGGC	700 GGCGGTCCAT
	0	720	730	740	750	760	770
CCGCCAAC	GC	TTCTGCCTCA	AGGGTGCTGC	CACCCTTACT	ATCCTTCTGG	GGATTTTCTT	CCTGTGCTGG
78		790	800	810	820	830	840
GCCCCCTT	CT	TCCTGCATCT	CTTGCTCATC	GTCCTCTGCC	CTCAGCACCC	CACCTGCAGC	TGCATCTTCA
ACAACTTO		860 CCTCTTC	870	880	890	900	910
			CTCCTCATCG	TCCTCAGCTC	CACIGITGAC	CCCCTCATCT	ATGCTTTCCG
92 CAGCCAGG		930 CTCCGCATGA	940 CACTCAAGGA		960	970	980
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			1220 TCCTGAAGAG	1230 CTTCACCTCT	1240 CCCCACCTAC	1250 1 AGGCAACTCC	.260 TGCTCAAGCC

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# Figure 1B

10			40 CTGAGAACGA	50 CTTTTAAAAC		70 AGCTCCATTC
80	90	100	110	120 GACAGAGGCC	130	140
150	160	170	180	190	200	210
				GCAGGGAGGG 260		
220 TCTGAGAATC				GTCTGGACTG		
290 ACCTGTCCAG	300 CCAGGGAGAG		320 CAGATCTGGG	330 GGTGCCCAGA		350 CAGGCATGGG
360 GACACCCAAG		-		400 ACACCTGGAG		
				470 TATGGCTGTG		
200				540 CTGGGGCTGG		
570 CGGTGCCTGG			600 CTCTTCCTCA	610 GCCTGGGGCT	620 GGTGAGCTTG	630 GTGGAGAACG
				680 CTCACCCATG		
710 GGCCTTGTCG				750 GAGACGGCCG		770 GCTGGAGGCC
780 GGTGCACTGG				820 ACAATGTCAT		
850 CCATGCTGTC				890 GGACCGCTAC		
920 GCGCTACCAC	930 AGCATCGTGA	940 CCCTGCCGCG	950 GGCGCCGCGA	960 GCCCTTGCGG	970 CCATCTGGGT	980 GGCCAGTGTC
990 GTCTTCAGCA	1000 CGCTCTTCAT	1010 CGCCTACTAC	1020 GACCACGTGG	1030 CCGTCCTGCT	1040 GTGCCTCGTG	1050 GTCTTCTTCC
1060 TGGCTATGCT	1070 GGTGCTCATG			1100 GCTGGCCCGG	1110 GCCTGCCAGC	1120 ACGCCCAGGG
	1140 CTCCACAAGA				1180 TTAAAGGCGG	1190 TGTCACCCTC
1200 ACCATCCTGC	1210 TGGGCATTTT	1220 CTTCCTCTGC	1230 TGGGGCCCCT	1240 TCTTCCTGCA	1250 TCTCACACTO	1260 ATCGTCCTCT

# Figure 1C

1270	1280	1290	1300	1310	1320	1330
GCCCCGAGCA	CCCCACGTGC	GGCTGCATCT	TCAAGAACTT	CAACCTCTTT	CTCGCCCTCA	TCATCTGCAA
1340	1350	1360	1370	1380	1300	1.00
						1400
TGCCATCATC	GACCCCCTCA	TCTACGCCTT	CCACAGCCAG	GAGCTCCGCA	GGACGCTCAA	GGAGGTGCTC
1410	1420	1430	1440	1450	1460	1470
ACATGCTCCT	GGTGAGCGCG	GTGCACGCGC	TTTAAGTGTG	CTGGGCAGAG	GGAGGTGGTG	ATATTCTCCT
1480	1490	1500	1510	1520	1530	1540
CTGGTTCCTG	TGTGACCCTG	GGCAGTTCCT	TACCTCCCTG	GTCCCCGTTT	GTCAAAGAGG	ATGGACTAAA
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Figure 2B	mouse MSH-R	human MSH-R	human ACTH-R	rat cannab.	mouse MSH-R	human MSH-R	human ACTH-R	rat cannab.	mouse MSH-R	human MSH-R	human ACTH-R	rat cannab.

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Figure 2D	mouse MSH-R	human MSH-R	human ACTH-R	rat cannab.		mouse MSH-R	human MSH-R	human ACTH-R	rat cannab.		mouse MSH-R	human MSH-R	human ACTH-R	rat cannab.

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Figure 2E	mouse MSH-R	human MSH-R	human ACTH-R	rat cannab.		mouse MSH-R	human MSH-R	human ACTH-R	rat cannab.		mouse MSH-R	human MSH-R	human ACTH-R	rat cannab.	

Figure 3

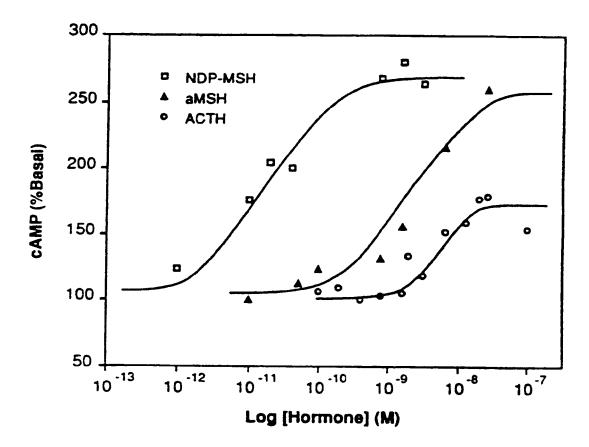


Fig. 4

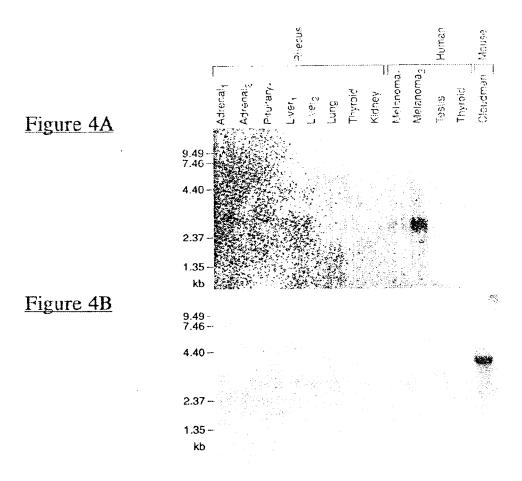
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PCT/US 93/03247

International Application No

I CLASSIE	CATION OF SURIE	CT MATTER (if several classification s	symbols apply, indicate ail)					
1. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC								
			C12P21/08; C12	N5/10				
Int.CI.	5 C12N15/12	2; C07K13/00; 2; A61K37/02;	A61K39/395; C12	201/68				
	C12N15/62	2; ADIK3//U2;	A01K39/393, C18	Q17 08				
II. FIELDS	SEARCHED							
		Minimum Docum	entation Searched <sup>7</sup>					
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		Documentation Searched other to the Extent that such Documents	r than Minimum Documentation are Included in the Fields Searched <sup>8</sup>					
<u> </u>		ED TO BE RELEVANT <sup>9</sup> ocument, <sup>11</sup> with indication, where approp	since of the relativist passages 12	Relevant to Claim No.13				
Category °	Citation of D	ocument, with indication, where approp	Hate, of the felevant passages					
P,X	SCIENCE		040750 04	1-45				
	vol. 257, 28 August 1992, LANCASTER, PA pages 1248 - 1251 Mountjoy KG; Robbins LS; Mortrud MT; Cone RD;  ¹The cloning of a family of genes that encode the melanocortin receptors.¹ see the whole document							
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coment, with indication, where appropriate, of the relevant passages	Relevant to Claim No
IICAL JOURNAL September 1992, LONDON, GB 382 ivier GW; Adams G; Erskine RG; Branch SK; Moss SH; Notarianni W; 'Isolation and partial n of a melanocyte-stimulating eptor from B16 murine melanoma vel approach using a cleavable d photoactivated ligand and n-coated magnetic beads.' le document	1-45
CELLULAR PHYSIOLOGY o. 1, October 1988, WILEY-LISS,  44  Montague PM; Hearing VJ; of melanocyte stimulating eptors correlates with igmentation, and can be y interferons.'	1-45
JRNAL OF PHARMACOLOGY  o. 1-2, 31 May 1990,  32  ty NB; Schmidt-Sole J; Piterman alomon Y; 'The melanocortin the rat lacrimal gland: a m for the study of MSH stimulating hormone) as a eurotransmitter.'  le document	1-45
	September 1992, LONDON, GB 382  ivier GW; Adams G; Erskine RG; Branch SK; Moss SH; Notarianni W; 'Isolation and partial n of a melanocyte-stimulating eptor from B16 murine melanoma vel approach using a cleavable d photoactivated ligand and n-coated magnetic beads.' le document  CELLULAR PHYSIOLOGY o. 1, October 1988, WILEY-LISS,  44 Montague PM; Hearing VJ; of melanocyte stimulating eptors correlates with igmentation, and can be y interferons.' le document  JRNAL OF PHARMACOLOGY o. 1-2, 31 May 1990, 32 ty NB; Schmidt-Sole J; Piterman alomon Y; 'The melanocortin the rat lacrimal gland: a n for the study of MSH stimulating hormone) as a eurotransmitter.'

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